Universidade Federal de Mato Grosso do Sul
Programa de Pós-graduação em Saúde e Desenvolvimento na Região Centro-Oeste

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AÇÃO DE AGENTES IMUNOMODULADORES EM CÉLULAS MONONUCLEARES DE COLOSTRO EM CO-CULTURA COM CÉLULAS DE TUMOR DE MAMA

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Tese apresentada como requisito para obtenção do título de doutor(a) pelo Programa de Pós-graduação em Saúde e Desenvolvimento na Região Centro-Oeste da Universidade Federal de Mato Grosso do Sul.
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Campo Grande
2018

Dedico este trabalho à minha família, meus pais e irmãs e ao meu amado esposo pelo imenso incentivo. Agradeço à Deus por todas as conquistas.

"O período de maior ganho em conhecimento e experiência é o período mais difícil da vida de alguém."

Dalai Lama

Agradecimentos

Sou grata primeiramente à Deus, por todas as bênçãos recebidas e que ainda continuarei a receber.

À minha família pela força, companheirismo e por incentivar a minha busca pelo conhecimento. Agradeço, aos meus pais Ascindino João da Silva e Odete Trindade Álvares, por toda sua dedicação e amor dados a mim e à minha irmãs. Agradeço às minhas irmãs Renatty Graziele Trindade da Silva e Rayza Trindade da Silva, pelo apoio nos momentos que eu quis desanimar, mesmo estando longe. À minha avó Maria Muniz da Silva pelo seu carinho e orações. Ao meu amadíssimo esposo Tiago Souza Fernandes, pelo companheirismo, amizade e dedicação, nos momentos bons e ruins, me incentivando a seguir adiante todos os dias, me dando amparo e amor. Agradeço à família do meu esposo pelo incentivo dado aos meus estudos.

Agradeço à minha orientadora Profª.Dra. Inês Aparecida Tozetti, pela orientação e confiança de me orientar mesmo antes de me conheçer. Agradeço à minha co-orientadora Profª. Dra. Adenilda Cristina Honório-França, que desde à graduação está presente na minha vida acadêmica, pela orientação, pela confiança, pela oportunidade de ser sua orientanda, muito obrigada de coração. Agradeço também ao querido Prof. Dr. Eduardo Luzía França que com seu ânimo, incentivo e autoestima, nunca deixa seus alunos desanimarem, mesmo nos momentos mais difíceis, nos ajudando no desenrolar do nosso trabalho da melhora maneira.

Agradeço ao Programa de Pós-graduação em Saúde e Desenvolvimento na Região Centro-Oeste da Universidade Federal de Mato Grosso do Sul.

Quero agradecer aos meus amigos Danny Laura Gomes Fagundes, Cláudia Cristina de Souza Pereira, Alessandra Lima Deluque, Mahmi Fujimori, Patrícia Gelli Feres de Marchi , Aron Carlos de Melo Cotrim, José Nivaldo, Maraisa Delmut Borges, Kellen Menezes, Adriele Ataides de Queiroz, Tassiane Moraes, Renata, Suiane, ao companheirismo vivenciados todos esse anos.

Agradeço a todos os companheiros do Laboratório de Imunomodulação e Saúde Materno-Infantil e Croimunomodulação, pela ajuda, pelos momentos de descontração e pela união dentro do laboratório. Agradeço também ao colegas e professores do LABIMUNOBIO InBio/UFMS que me acolheram tão bem.

Agradeço ao Hospital e Pronto Socorro Municipal de Barra do Garças por abrirem suas portas para que pudéssemos fazer o primeiro contato com as puérperas.

Agradeço imensamente a todas as voluntárias desta pesquisa, pela generosidade da doação de amostras de leite de materno.

À Universidade Federal de Mato Grosso do Sul e à Universidade Federal de Mato Grosso, a todos os professores e funcionários destas instituições, por todas as contribuições prestadas em benefício da minha formação.

Ao CNPQ pelo apoio financeiro dado e o incentivo à pesquisa.

À Banca examinadora pela contribuição para a melhoria deste trabalho e a todos que de alguma forma contribuíram e que não foram anteriormente citados.

A todos vocês, meus sinceros agradecimentos!

Resumo

O Câncer de mama é um importante problema de saúde pública e até o ano de 2050 serão cerca de 3,2 milhões de casos novo no mundo. A dieta tem um papel fundamental na etiologia do câncer e a amamentação associa-se a uma menor incidência de câncer de mama. O colostro humano é rico em componentes solúveis e celulares como fagócitos, imunoglobulinas, citocinas e quimiciocinas, além de hormônios. O objetivo deste estudo foi investigar o efeito de imunoglobulina A secretória (SIgA) e melatonina (MLT) adsorvidas em microesferas de polietilenoglicol (PEG) em células mononucleares de colostro humano (MN) co-cultivadas com linhagens de células de tumor de mama (MCF-7). A coleta do colostro foi realizada no período diurno para os testes com SIgA, e nos períodos diurno e noturno para as análises com MLT. Células MN, células MCF-7 e co-cultura (células MN e MCF-7) foram pré-incubadas por 24 h com SIgA ou MLT, PEG e/ou PEG-SIgA e PEG-MLT. Na presença de SIgA adsorvida ou não a microesfera de PEG foram avaliados em células MN do colostro, células MCF-7 e co-cultura a fenotipagem celular, concentração de quimiocinas no sobrenadante de cultura, viabilidade celular, liberação de cálcio intracelular e os índices de apoptose. Na presença de melatonina adsorvida ou não a microesfera de PEG em duas fases (diurno e noturno), avaliou-se: o estresse oxidativo, a liberação de cálcio intracelular e a apoptose. Observou-se que na co-cultura na quando incubadas com PEG-SIgA houve aumento da expressão de células $TCD8^{+}$, aumento na liberação das quimiocinas MIG e redução de IL-8 e MCP-1. As células MN em co-cultura com as células MCF-7, independentemente do tratamento, apresentaram a maior liberação de Ca^{2+} intracelular e apoptose. Na presença de melatonina adsorvida a microesfera de PEG, em fase noturna, a co-cultura apresentou aumento da liberação de superóxido. As células MN tratadas com MLT, independete da hora do dia, exibiram altas concentrações da enzima superóxido dismutase (Cu-Zn SOD) em sobrenadante da cultura, enquanto que as células MCF-7 apresentaram níveis elevados desta enzima quando incubadas com PEG-MLT durante a fase noturna. A co-cultura no período noturno, independente do tratamento, mostrou níveis maiores da enzima. A maior quantidade de liberação intracelular de Ca^{2+} foi observada em células MN e células MCF-7 em co-cultura quando tratadas por PEG-MLT durante o período noturno. Independentemente da fase do dia, observou-se índices maiores de apoptose apresentados em co-cultura de células tratadas com PEG-MLT. Esses dados sugerem que a SIgA associada a microesfera de PEG foi capaz de aumentar a expressão de células $TCD8^{+}$, controlar a liberação de quimiocinas e aumentar o Ca^{2+} e a apoptose intracelular em co-cultura podendo ser um mecanismo importante do sistema imunológico com atividade antitumoral, enquanto que a liberação modificada de melatonina associada aos efeitos deste hormônio produzido fisiologicamente em maiores concentrações durante a noite pode controlar o estresse oxidativo e aumentar a atividade efetiva desse hormônio contra tumores.

Palavras Chaves: células MCF-7, células mononucleares de colostro, melatonina, microesfera de polietilenoglicol, SIgA.

Abstract

Breast cancer is a major public health problem and by the year 2050 will be about 3.2 million new cases in the world. Diet plays a key role in the etiology of breast cancer and breastfeeding is associated with a lower incidence of breast cancer. Human colostrum is rich in soluble and cellular components such as phagocytes, immunoglobulins, cytokines and chemokines, as well as hormones. The objective of this study was to investigate the effect of secreted immunoglobulin A (SIgA) and melatonin (MLT) adsorbed on polyethylene glycol (PEG) microspheres in human colostrum (MN) mononuclear cells co-cultured with breast tumor cell lines (MCF -7). Colostrum collection was performed in the daytime for SIgA tests, and during daytime and night time for MLT analyzes. MN cells, MCF-7 cells and co-culture (MN and MCF-7 cells) were preincubated for 24 h with SIgA or MLT, PEG and / or PEG-SIgA and PEG-MLT. In the presence of SIgA adsorbed or not the PEG microsphere, cell phenotyping, concentration of chemokines in the culture supernatant, cell viability, intracellular calcium release, and colony counts were evaluated in MN cells from colostrum, MCF-7 cells and co-culture. apoptosis. In the presence of melatonin adsorbed or not the PEG microsphere in two phases (diurnal and nocturnal), we evaluated: oxidative stress, release of intracellular calcium and apoptosis. It was observed that in the co-culture when incubated with PEG-SIgA there was an increase in the expression of CD8 + T cells, increase in the release of the MIG chemokines and reduction of IL-8 and MCP-1. MN cells in co-culture with MCF-7 cells, regardless of treatment, had the highest release of intracellular Ca2 + and apoptosis. In the presence of melatonin adsorbed to the PEG microsphere in the night phase, the co-culture increased the release of superoxide. MLT-treated MN cells, regardless of time of day, exhibited high concentrations of the superoxide dismutase (Cu-Zn SOD) enzyme in culture supernatant, whereas MCF-7 cells showed high levels of this enzyme when incubated with PEG-MLT during the night phase. Co-culture at night, regardless of treatment, showed higher levels of the enzyme. The greater amount of intracellular Ca 2+ release was observed in MN cells and MCF-7 cells in co-culture when treated by PEG-MLT during the night time. Regardless of the phase of the day, higher rates of apoptosis were observed in co-culture of cells treated with PEG-MLT. These data suggest that SIgA associated with the PEG microsphere was able to increase the expression of CD8 + T cells, to control the release of chemokines and to increase Ca2 + and intracellular apoptosis in co-culture, being an important mechanism of the immune system with antitumor activity, while the modified release of melatonin associated with the effects of this hormone produced physiologically at higher concentrations at night can control oxidative stress and increase the effective activity of this hormone against tumors.

Key Words: MCF-7 cells, colostrum mononuclear cells, melatonin, polyethylene glycol microsphere, SIgA.

LISTA DE ABREVIATURAS E SIGLAS

ANOVA: análise de variância

ATCC: American Type Culture Collection

Bak: Membro pró-apoptótico da família Bcl-2

Bax: Proteína X associada à Bcl-2

Bcl-2: Linfoma 2 de célula B (B cell lymphoma 2)

Bcl-XL**:** B-cell lymphoma-extra large

BRCA1: breast cancer 1, early onset

BRCA2: breast cancer 2, early onset

BSA: soro albumina bovino

CAT: enzima catalase

CCL2/MCP-1: proteína quimioatraente de macrófagos e monócitos

CCL5/RANTES: proteína secretada e regulada pela ativação de linfócitos T

CD: Cluster of Differentiation

CEP: Comitê de Ética e Pesquisa

CNPq: Conselho Nacional de Desenvolvimento Científico e Tecnológico

CO_2: dióxido de carbono

CONEP: Conselho Nacional de ética em Pesquisa

Cu-ZnSOD: enzima cobre-zinco Superóxido Dismutase

CXCL10/ IP-10: proteína induzida por interferon-gama

CXCL8/IL-8: interleucina-8

CXCL8/IL-8: proteína quimioatraente de neutrófilo

CXCL9/MIG: molécula induzida por interferon-gamma por células mononucleares estimuladas

DAF: diacetato de fluoresceína

DLS: Dynamic Light Scattering

DMEM: Dulbecco's Modified Eagle Medium)

DMSO: Dimetilsulfóxido

DNA: ácido desoxirribonucleico

DO: Densidade óptica

EC-SOD: Enzima Extracelular Superóxido dismutase

EDTA: Ácido etilenodiamino tetra-acético

EGF: Fator de crecimento epidermal

EGF-R: receptor de fator de crescimento epidermal

ELISA: Enzyme-Linked Immunosorbent Assay

FcαR: receptor Fc para cadeia α

FITC: Fluorescein isothiocyanate

Fluo-3: Fluo 3 Acetoxymethyl

GPXs: glutationa Peroxidase

H_2O_2: peróxido de hidrogênio

Ham's F10: meio de cultura de células de Ovário de Hamster Chinês (CHO).

HEPES: Ácido 4-(2-hidroxietil) -1-piperazina etanosulfônico

IFN-γ: interferon gama

SIgA: imunoglobulina A secretória

IgG: imunoglobulina G

IgM: imunoglobulina M

IL-2: interleucina 2

IP: iodeto de propídeo

MALT: Tecido Linfoide Associado à Mucosa

MCF-7: linhagem celular de adenocarcinoma de mama humano

MLT: melatonina

MN: células mononucleares

Mn-SOD: enzima Manganês Superóxido Dismutase

MT1: receptor de melatonina 1

NBT: azul de nitrotetrazólio

NK: natural killer cell

NO: óxido nítrico

O_2^-: ânion superóxido

$O_{2:}$ oxigênio molecular

OH^{-}: radical hidroxila

PBS: Tampão fosfato salino

PBS-BSA: PBS acrescido de 0,1% de Soro-Albumina Bovina

PE: ficoeritrina

PEG: polietilenoglicol

RNA: ácido ribonucleico

RNS: espécies reativas de nitrogênio

ROS: espécies reativas de oxigênio

RPMI: roswell park memorial institute

RSS: espécies reativas de enxofre

rTNF: receptor de fator de necrose tumoral

SFB: soro fetal bovino

SIDA: acquired immunodeficiency syndrome

SIgA: imunoglobulina A secretória

SOD: enzima superóxido dismutase

$TCD4^{+}$: linfóticos T helper

$TCD8^{+}$: linfócitos T citotóxicos

TCLE: Termo de Consentimento Livre e Esclarecido

TNF-α: fator de necrose tumoral alfa

UV: ultravioleta

Sumário

1. INTRODUÇÃO

O câncer de mama é crescente em todo o mundo, e está diretamente relacionado ao aumento do tempo de vida, de exposição a fatores de risco, além das mudanças de hábitos. O câncer de mama é uma doença multifatorial e na maioria das vezes afeta principalmente as mulheres, podendo levar à morte (GHONCHEH; POURNAMDAR; SALEHINIYA, 2016).

O câncer de mama está intimamente relacionado a um desequilíbrio entre espécies reativas de oxigênio e enzimas antioxidantes, porém os mecanismos pelos quais isso ocorre ainda não são bem compreendidos. O balanço entre o dano oxidativo e a proteção antioxidante é imprescindível para que ocorra um equilíbrio no organismo. A eliminação inadequada de radicais livres leva ao estresse oxidativo, que favorece o surgimento de diversas doenças, entre estas o câncer (TAS et al., 2005). O câncer e a inflamação crônica também estão intimamente ligados, e a maioria das doenças crônicas, são beneficiadas pelo desequilíbrio oxidativo (REUTER et al., 2010). Sendo assim o controle da produção de espécies reativas, bem como a manutenção do equilíbrio oxidativo são primordiais para o controle da progressão de tumores.

Alguns fatores como estilo de vida saudável e aleitamento materno estão relacionados à prevenção do câncer de mama (GRADIM et al., 2011). O leite materno contém vários componentes solúveis e celulares, como fagócitos, imunoglobulinas e hormônios, entre estes se destacam a IgA secretória (SIgA) e o melatonina (MLT) (HONORIO-FRANÇA et al., 1997, FRANÇA et al., 2009; MORCELI et al., 2013).

O colostro difere de outras secreções, pois contém grandes quantidades de leucócitos viáveis comparáveis aos encontrados no sangue e atuam como mediadores anti-inflamatórios (HONORIO-FRANÇA et al., 1997; 2001). Acredita-se que tanto os componentes solúveis como celulares interagem entre si e podem ser importantes para a imunidade antitumoral.

A presença de SIgA na secreção confere papel protetor para várias doenças. É a principal classe de anticorpos no leite materno humano e sua atividade provavelmente parece estar envolvida no efeito protetor da amamentação que contribui para a redução da incidência do câncer de mama (HONORIO-FRANÇA et al., 2016).

Assim como a SIgA, a melatonina, um hormônio produzido pela glândula pineal, está envolvida em vários processos fisiológicos, inclusive na regulação funcional do leite

materno. A melatonina se relaciona com efeitos antioxidantes e anti-inflamatórios (FRANÇA-BOTELHO et al., 2011) e efeitos pró-oxidantes, de suma importância no equilíbrio do estresse oxidativo como mecanismo de proteção, a depender do ambiente em que ela irá atuar (KORKMAZ, 2009).

Alguns estudos têm reportado que a biodisponibilidade, bioviabilidade e a biofuncionalidade, tanto de SIgA como da melatonina, podem ser potencializadas quando associadas em sistemas de liberação modificada (FRANÇA et al, 2015; HONÓRIO-FRANÇA et al., 2016). Entre estes sistemas, as microesferas de polietilenoglicol (PEG) tem sido consideradas como um importante veículo para a administração de várias drogas, produtos naturais e hormônios (SCHERER et al, 2011; REINAQUE et al., 2012; FAGUNDES et al., 2012; GUIMARÃES et al., 2013; HARA et al., 2013).

A administração de drogas adsorvidas a sistemas carreadores, como as microesferas de PEG tem sido um tratamento alternativo para várias doenças (JEVSEVAR et al., 2010). Esses sistemas de liberação são agentes promissores para a entrega do hormônio melatonina (FRANÇA et al., 2015) e anticorpo como SIgA (HONORIO-FRANÇA et al., 2016), prevenindo a degradação das proteínas por enzimas metabólicas e principalmente aumentando a sua biodisponibilidade no organismo (YU et al., 2005).

O controle do processo da carcinogênese está estreitamente relacionado com o controle de apoptose, uma vez que a célula tumoral tem esse sistema alterado, favorecendo sua proliferação e promoção (HANAHAN; WEINBERG, 2011). Deste modo, tratamentos de câncer são direcionados à indução do aumento de apoptose das células tumorais (NICHOLSON, 2000; WONG, 2011), o que pode ser favorecido pela utilização de agentes imunomoduladores como SIgA e MLT para atuação nas células imunocompetentes e auxiliar na erradicação do tumor.

Assim, o objetivo do presente trabalho foi investigar a influência dos componentes solúveis e celulares presentes no colostro humano (SIgA, MLT e células mononucleares) como agentes imunomoduladores em associação com micoesferas de PEG frente a células de tumor de mama, como possíveis agentes para uso terapêutico no tratamento de câncer de mama.

2. REVISÃO DA LITERATURA

2.1 Aspectos epidemiológicos do câncer de mama

O Câncer de mama é um importante problema de saúde pública, com cerca de 59.700 de casos novos no Brasil, no ano de 2018 (INCA, 2018) e estimativa de que 627.000 de mulheres morrerão de câncer de mama no mundo em 2018, o que equivale a 15% de todas as mortes por câncer entre as mulheres (WHO, 2018).

A incidência mundial e mortalidade por esta doença, estão altamente relacionadas e estima-se que até o ano de 2050 surgirão cerca de 3,2 milhões de casos novos de câncer de mama no mundo. Apesar dos avanços tecnológicos, ainda existem vários mecanismos que devem ser elucidados na direção da erradicação desta doença que acomete a população mundial (TAO et al., 2015).

O câncer de mama é a maior causa de morte por câncer nas mulheres em todo o mundo, seguido pelo câncer de colo do útero. É a segunda causa de morte por câncer nos países desenvolvidos, atrás somente do câncer de pulmão, e a maior causa de morte por câncer nos países em desenvolvimento (INCA, 2016).

O Brasil vem sofrendo mudanças em seu perfil demográfico, consequência, entre outros fatores, do processo de urbanização populacional, da industrialização e dos avanços da ciência e da tecnologia. A essas novas caraterísticas da sociedade brasileira, unem-se os novos estilos de vida e a exposição, ainda mais intensa, a fatores de risco próprios do mundo contemporâneo. Essas mudanças trouxeram alterações no perfil de morbimortalidade, diminuindo a ocorrência das doenças infectocontagiosas e colocando as doenças crônico-degenerativas novamente no centro, e por esse motivo vem, progressivamente, sendo acrescentado ao repertório tradicional da vigilância epidemiológica (INCA, 2016).

A estimativa é de que no biênio 2018-2019, no Brasil terá cerca de 600 mil casos novos de câncer, muito semelhante aos dados da América Latina onde os cânceres mais frequentes em homens será de próstata (61 mil) e em mulheres o câncer de mama (58 mil).. Esse tipo de câncer, com exceção do câncer de pele não melanoma, é o mais frequente nas mulheres das Regiões Sul (74,30/ 100 mil), Sudeste (68,08/ 100 mil), Centro-Oeste (55,87/ 100 mil) e Nordeste (38,74/ 100 mil). Na região Norte, é o

segundo tumor mais incidente (22,26/ 100 mil). Para os anos de 2014 e 2015 estimou-se para o estado de Mato Grosso (610/ 100 mil), Goiás (1500/ 100 mil), Distrito federal (920 / 100mil) e Mato Grosso do Sul (770/ 100 mil) casos de câncer de mama (INCA, 2018).

Alguns fatores de risco para o desenvolvimento do câncer de mama são bem conhecidos, como: envelhecimento, história familiar de câncer de mama, consumo de álcool, excesso de peso, sedentarismo, exposição à radiação ionizante e alta densidade do tecido mamário (INCA, 2016).

É uma doença em que fatores biológico-endócrinos, vida reprodutiva, comportamento e estilo de vida estão altamente relacionados, e a idade ainda é um dos fatores de risco mais importantes. A condição de nunca ter tido um filho ou ter o primeiro filho após os 30 anos de idade contribuem para o aumento no risco do câncer de mama. Por outro lado, a amamentação, prática de atividades físicas, alimentação saudável com a manutenção do peso corporal, estão associadas a um menor risco de desenvolver esse tipo de câncer (INCA, 2016).

Diferentes fatores genéticos como a variabilidade na frequência da mutação dos genes BRCA1 (breast cancer 1, early onset) e BRCA2 (breast cancer 1, early onset), supressores do câncer, podem estar diretamente relacionados quanto as diferenças encontrada entre os diferentes países do mundo. Durante a década de 80, houve um aumento no número de casos de câncer no Estados Unidos, sendo que só após a década de 90 , com campanhas em torno da mamografia, onde puderam detectar e tratar os casos que houve diminuição da incidência da mortalidade por câncer de mama (HULKA; MOORMAN, 2001).

No Brasil, o Ministério da Saúde preconiza a mamografia bienal para mulheres entre 50 a 69 anos, porém as que possuem risco elevado de câncer de mama, com histórico familiar, o que se recomenda é um acompanhamento individualizado, pois mesmo tendo bom prognóstico, se detectado a tempo, ainda assim, se relaciona com alto índice de morte (INCA, 2016).

O câncer de mama é a neoplasia mais temida pelas mulheres, uma vez que a sua ocorrência causa grande impacto psicológico, funcional e social, atuando negativamente nas questões relacionadas à autoimagem, convívio social e à percepção da sexualidade. É considerado de grande importância na assistência à saúde da mulher, devido à elevada prevalência, morbidade e mortalidade (PINHEIRO et al., 2013).

Estudos tem revelado que o risco relativo de se ter câncer de mama reduz cerca de 7,0% para cada filho nascido e cerca de 4,3 %, para cada 12 meses de amamentação. O que sugere que quanto maior a duração do período do aleitamento materno, maior a proteção conferida por essa prática contra o câncer de mama (ROMIEU et al., 1996; IP et al., 2007).

A vida moderna tem favorecido o desenvolvimento de câncer de mama, hoje em dia, muitas mulheres não amamentam seus filhos ou amamentam por menos tempo, devido o retorno aos seus empregos. A academia Americana de Pediatria e a Organização Mundial da Saúde (OMS) recomendam amamentação exclusiva, com leite materno, nos primeiros seis meses de vida de nascimento dos bebês, porém o que se percebe é que cada vez mais as mães têm amamentado por pouco tempo e as que ainda amamentam acabam introduzindo fórmulas prontas na dieta dos recém-nascidos antes de completarem 6 meses de vida. A amamentação reduz o risco de câncer, pois além de diminuir os ciclos menstruais, deixando o organismo das mães com menor ação dos estrógenos, também amadurecem as células da mama protegendo essas células contra possíveis alterações na involução da mama no período pós amamentação (LIPWORTH; BAILEY; TRICHOPOULOS, 2000).

2.2 Câncer de mama

As células tumorais surgem a partir de mutações que ocorrem no DNA (ácido desoxirribonucleico) ou RNA (ácido ribonucleico), que podem ser desencadeadas por radiação, bactérias, fungos, vírus, produtos químicos, etc. O câncer se desenvolve quando o sistema imune está alterado e a produção de células cancerígenas é muito maior que a de células de defesa do próprio organismo, ou seja, o organismo debilitado por dieta pobre, predisposição genética e idade avançada conduz a um ambiente perfeito para o desenvolvimento do câncer (SHARMA et al.,2010).

A estrutura da mama é constituída de tecidos glandulares compostos pelas glândulas produtoras de leite e os ductos por onde passa o leite produzido e tecidos estromados, os quais são tecidos conjuntivos gordurosos e fibrosos. Além desses tecidos a mama também é composta pelo tecido do sistema imunológico e sistema linfático (SHARMA et al., 2010). Para que ocorra um desenvolvimento normal do tecido da mama é necessário que haja um equilíbrio entre proliferação celular e apoptose.

Enquanto que no crescimento tumoral ocorre o inverso, há uma redução da apoptose e o aumento da proliferação (PARTON; DOWSETT; SMITH, 2001).

O câncer de mama, é considerado uma doença heterogênea, quanto à sua morfologia e à clínica, ocorre a partir de uma proliferação desordenada das células do tecido mamário, porém cerca de 80% dos tipos de tumores de mama origina-se no epitélio ductal, conhecido como carcinoma ductal invasivo (BRASIL, 2016). Os carcinomas podem ser invasivos ou *in situ*. Os carcinomas invasivos, são assim chamados pois tem alto potencial metastático, já os carcinomas *in situ* tem baixo potencial metastáticos e podem surgir tanto em lóbulos como em ductos mamários (RICHIE; SWANSON, 2003). Durante a carcinogênese, as mutações genéticas vão se acumulando e o fenótipo das células vão se modificando através de lesões malignas, evoluindo para o câncer invasivo (PARTON; DOWSETT; SMITH, 2001).

O sistema imunológico tem a capacidade de controlar e identificar tumores, porém os tumores desenvolvem mecanismos de escape contra a vigilância imunológica. A chamada “imunoedição” seria capaz de alterar o perfil antigênico dos tumores, contudo as mutações das células neoplásicas levam a uma resistência a esse tipo de ação do sistema imunológico levando à evasão do tumor (DUNN et al., 2002).

Dentre as mutações, as alterações mais importantes que ocorrem são apoptose diminuída, diferenciação celular reduzida, angiogênese aumentada, produção de fatores de crescimento, menor dependência de fatores de crescimento externos, aumento na secreção de proteases, menor inibição celular por densidade populacional em cultura, menor interação célula-célula e célula-matriz extracelular (HANAHAN; WEINBERG, 2011; SERRANO; THEODORO; PINHAL, 2014).

Os tratamentos utilizados nos pacientes com câncer de mama têm como objetivos principais a cura, o prolongamento do tempo de vida e melhoraria da qualidade de vida, levando em conta principalmente a questão psicológica das mulheres afetadas. A cirurgia é um dos primeiros recursos que pode ser utilizado, e depende do estadiamento do tumor. Pode ser definida dependendo do tipo histológico, podendo ser conservadora (retirada de apenas uma parte da mama) ou não conservadora (mastectomia da mama), e pode ser precedida ou completada com radioterapia e quimioterapia. A radioterapia é utilizada com o objetivo de destruir as células remanescentes após a cirurgia ou para reduzir o tamanho do tumor antes da cirurgia. É um método capaz de destruir as células tumorais pelo emprego de radiações ionizantes, atua local ou regionalmente, podendo

ser indicada de forma exclusiva ou associada a outros métodos terapêuticos (BRASIL, 2006).

A quimioterapia, tratamento sistêmico, utiliza quimioterápicos que são compostos químicos que atuam diminuindo a multiplicação celular e, consequentemente, a expansão dos tumores, utilizam-se substâncias citotóxicas, isoladas ou combinadas. Os quimioterápicos afetam também as células normais, porém o maior dano ocorre nas células malignas. Os efeitos colaterais não hematológicos são toxicidades gastrintestinais, cardíacas, renais, pulmonares, hepáticas, neurológicas, dermatológicas, alterações metabólicas, disfunções reprodutivas e reações alérgicas; o grupo de toxicidade hematológica engloba a leucopenia, a trombocitopenia e a neutropenia (AGUIAR et al., 2008)

Já a hormonioterapia consiste na admininstração de hormônios que impedem o crescimento das células tumorais, utilizada em pacientes que têm tumores que expressam receptores hormonais para estrógeno e progesterona. O tamoxifeno, um medicamento modulador seletivo do receptor de estrogênio , tem um lugar estabelecido na prevenção e no tratamento do câncer de mama, mas devido a seus efeitos colaterais, drogas de nova geração têm sido introduzidas na tentativa de substituir o tamoxifeno na prática clínica (ABDULKAREEM; ZURMI, 2012).

A imunoterapia consiste em um processo imunomodulador, é a utilização de substâncias que modificam a resposta do sistema imunológico do organismo (ABBAS; LICHTMAN; PILLAI, 2011b; BRASIL, 2006), e visa aumentar a sensibilidade do sistema imunológico aos antígenos tumorais ou aumentar a eficácia das respostas imunológicas frente a esses antígenos (EGGERMONT, KROEMER e ZITVIGEL, 2013).

A resposta esperada pela quimioterapia, radioterapia, tratamentos hormonais, é a indução da apoptose, pois o equilíbrio entre proliferação e apoptose celular é determinante para seu crescimento (TAMM; SCHRIEVER; DORKEN, 2001).

Após o início da quimioterapia, dentro de 24 h, ocorre um aumento significativo na apoptose em tumores mamários, associado com uma diminuição da proliferação. Essa resposta pode ocorrer de maneira diferenciada, nos vários tipos de tumores, e ao final das quimioterapias ocorre aumento dos níveis da proteína antiapoptótica Bcl-2, o que favorece a ocorrência de células residuais quimiorresistentes, que poderão ser importantes para recidivas do tumor (PARTON; DOWSETT; SMITH, 2001)

2.3 Apoptose e Estresse Oxidativo

A apoptose pode ser definida como uma morte programada e regulada da célula, não patológica, ou seja, um fenômeno fisiológico(LOCKSHIN; WILLIANS, 1964).

Perturbações nas vias de sinalização de proliferação celular e morte programada podem levar a inúmeras doenças como câncer, AIDS (SIDA- acquired immunodeficiency syndrome), doenças autoimunes, doenças degenerativas, entre outras. Investigações sobre o câncer eram baseadas no estudo da proliferação celular, porém hoje já se sabe que a apoptose, está intimamente relacionada com o entendimento da progressão da doença e da tumorigênese e é requerida como mecanismo de defesa antineoplásica (RUDIN; THOMPSON, 1997). Estudos revelam que vários agentes quimioterápicos agem induzindo a apoptose de células neoplásicas, o que leva a uma intensa investigação dos mecanismos e vias de morte celular programada, bem como, sua utilização no tratamento do câncer (NICHOLSON, 2000).

A morte celular programada é ativada por duas vias de sinalização, que culminam na morte da célula. A via intrínseca ou mitocondrial é ativada por lesão celular, espécies reativas de oxigênio, deficiências de fatores de crescimento e de sinais de sobrevivência, danos ao DNA ou dobramento das proteínas. É regulada pela família de proteínas Bcl-2, sendo que alguns membros desta família são pró-apoptóticos (Bax e Bak) e outros são antiapoptóticos (Bcl-2 e Bcl-XL), que na presença de fatores de crescimento e outros sinais de sobrevivência, funcionam como reguladores da apoptose pela inibição de Bax e Bak. Estímulos ativam as proteínas pró-apoptóticas da família Bcl-2, que se oligomerizam induzindo a permeabilização mitocondrial e consequente liberação de moléculas pró-apoptóticas nela presentes, como o citocromo c e outras proteínas pró apoptóticas que ativam enzimas citoplasmáticas, as caspases, inicialmente a caspase 9 que então cliva uma série de outras caspases, ativando caspase 3, o que resulta em fragmentação do DNA nuclear, levando à morte apoptótica (RUDIN; THOMPSON, 1997; ABBAS; LICHTMAN; PILLAI, 2011a; NICHOLSON, 2000; GRIVICICH; REGNER; ROCHA, 2007).

A via extrínseca ou via de receptores de morte é ativada pela ligação de receptores na superfície da célula do grupo de receptores de membrana da superfamília dos receptores de fatores de necrose tumoral (rTNF). Os receptores são ocupados e ativam

proteínas adaptadoras citoplasmáticas, que clivam caspase 8, que ativa várias outras caspases, ativando caspase 3 e levando a célula à apoptose. Pode ocorrer também uma amplificação da sinalização de receptor de morte quando a caspase 8 cliva e ativa uma proteína pró-apoptótica que induz apoptose pela via mitocondrial. Ocorrem alterações bioquímicas na membrana plasmática, essas alterações são reconhecidas por fagócitos e os corpos apoptóticos são rapidamente fagocitados por macrófagos e removidos sem causar um processo inflamatório. Alterações nos componentes das vias apoptóticas e sua relação com a ocorrência do câncer, como mecanismo de escape, tem sido elucidados para o desenvolvimento de novas terapias e métodos de prevenção do câncer (RUDIN; THOMPSON, 1997; ABBAS; LICHTMAN; PILLAI, 2011a; NICHOLSON, 2000; GRIVICICH; REGNER; ROCHA, 2007).

O estresse oxidativo, a inflamação crônica e o câncer estão intimamente ligados. O estresse oxidativo pode levar à inflamação crônica, que por sua vez pode mediar a maioria das doenças crônicas como câncer, diabetes, doenças cardiovasculares, neurológicas e pulmonares (REUTER et al., 2010).

A inflamação que acompanha o desenvolvimento de tumores, se inicia como uma resposta inflamatória normal, que ocorre no organismo frente a alguma desordem, porém as células cancerígenas conseguem se aproveitar das lesões provocadas no tecido em sua própria vantagem (SHALAPOUR; KARIN, 2015).

O estresse oxidativo está envolvido no processo de carcinogênese em todas as etapas, na iniciação, espécies oxidativas danificam o DNA introduzindo mutações genéticas e alterações estruturais; na promoção, ocorre aumento da população celular iniciada, ou seja, proliferação com consequente diminuição da apoptose; já na progressão, as espécies oxidativas participam do desenvolvimento de crescimento irreversível do câncer (KLAUNIG et al., 1998; HANAHAN; WEINBERG, 2011).

Na respiração aeróbica, temos a atuação de várias enzimas que atuam nas mitocôndrias de células eucarióticas, são as principais fontes de estresse oxidativo produzido dentro das células, o que incluem, além de outras, principalmente enzimas do complexo do citocromo P450. Externamente outras fontes contribuem para o estresse oxidativo celular, como agentes químicos, radiação UV, sedentarismo, etc. Os produtos resultantes do metabolismo oxidativo são necessários para vários eventos celulares, como transdução de sinal, ativação enzimática e controle da atividade das caspases que é ativada durante a apoptose (SOSA et al.,2013).

As espécies reativas são altamente instáveis, possuem meia vida curtas variando de alguns nano segundos, compostos mais reativos, há alguns segundos e horas para compostos mais estáveis. Essas espécies reativas desencadeiam reações que resultam na oxidação de macromoléculas para atingir estabilidade em seus orbitais. São conhecidas quatro categorias de espécies reativas de acordo com seu átomo central: espécies reativas de enxofre (RSS), espécies reativas de cloreto, espécies reativas de oxigênio (ROS), espécies reativas de nitrogênio (RNS). As principais espécies reativas produzidas a partir de oxigênio molecular (O_2) são: ânion superóxido (O_2^-), peróxido de hidrogênio (H_2O_2) e radical hidroxila (OH^-) (BABIOR et al., 1973; ĎURAČKOVÁ, 2010).

O radical hidroxila é a espécie reativa mais prejudicial. Já o peróxido de hidrogênio não é um radical livre propriamente dito, mas pode ter efeitos deletérios sobre DNA, proteínas e lipídios em concentrações altas. A cadeia respiratória mitocondrial também produz óxido nítrico (NO), que em condições de hipóxia pode gerar outras espécies de nitrogênio reativo (RNS) que induzem a peroxidação lipídica excessiva, o que pode aumentar o risco de mutagênese (HALLIWELL, 2001; POYTON, 2009; SCHRAUFSTATTER, 1988).

Alterações na homeostase do organismo, seja através do aumento da produção de ROS ou diminuição da remoção, resulta em estresse oxidativo (LIMO´N-PACHECO; GONSEBATT, 2009). O dano celular que as ROS podem causar, dependem da sua concentração intracelular e do equilíbrio entres as espécies pró-oxidantes e antioxidantes (SOSA et al., 2013; VESKOUKIS et al., 2012), ou seja várias patologias podem estar relacionadas ao aumento da geração de radicais livre e/ou diminuição do nível de antioxidantes (KLAUNIG et al., 1998). Espécies reativas levam ao mau funcionamento no mecanismo de reparo do DNA, geram mutações e esse processo aumenta o envelhecimento e a carcinogênese (MATSUI et al., 2000). Além de estar relacionadas com várias outras doenças como Alzheimer, Parkinson, Esclerose lateral amiotrófica, doença cardiovascular, disfunções do sistema imunológico, alergias, diabetes, doenças inflamatórias como, por exemplo, artrite reumatoide, entre outras. Durante a fagocitose e o processo infamatório, células liberam mediadores químicos que aumentam os níveis de ROS e saturam os mecanismos antioxidantes, a defesa natural contra a ação desses agentes, o que afeta e danifica as células vizinhas (SOSA et al.,2013).

Antioxidantes são os mecanismos de defesa das células, na tentativa de eliminar as espécies reativas. Os antioxidantes endógenos incluem glutationa, ácido alfa-lipóico,

coenzima Q, ferritina, ácido úrico, bilirrubina, metalotioneína, l-carnitina, melatonina, catalase (CAT), glutationa peroxidases (GPXs), superóxido dismutase enzimática (SOD), entre outros. Alguns outros antioxidantes podem ser introduzidos ao organismo pela dieta como ácido ascórbico (vitamina C), tocoferol (vitamina E), betacaroteno (vitamina A), ácido lipóico, ácido úrico, glutationa e polifenóis (SOSA et al., 2013; YOSHIDA et al., 2003).

As SOD (enzima superóxido dismutase) foram as primeiras enzimas antioxidantes a serem caracterizadas, são metaloenzimas que estão distribuídas pelo organismo desempenhando papel de defesa celular contra espécies reativas de oxigênio. Existem três tipos diferentes desta enzima expressas em células humanas, a enzima cobre-zinco Superóxido Dismutase (Cu-ZnSOD), enzima Manganês Superóxido Dismutase (Mn-SOD) e extracelular- SOD (EC-SOD), todos os quais são capazes de dismutar ânion superóxido a peróxido de hidrogênio (H_2O_2) e moléculas oxigênio (O_2) (REUTER et al., 2010).

Em praticamente todos os tumores, foram encontradas quantidades diminuídas de superóxido dismutase contendo manganês. Já as quantidades de superóxido dismutase contendo cobre e zinco, estavam reduzidas em muitos tumores, mas não em todos. Nestes casos a relação SOD e ânion superóxido estará em desequilíbrio, momento em que ocorre o dano celular (OBERLEY; BUETHER,1979).

2.4 Câncer de mama e amamentação

O equilíbrio entre uma resposta citotóxica de proteção e uma resposta não protetora pode ser regulado pelo estado imunológico geral do indivíduo (DENARDO et al., 2007). Compreender as funções efetoras imunes nos diferentes estágios de metástase do tumor é fundamental para a intervenção imune eficaz.

Um grande desafio para a pesquisa de tumores tem sido a identificação de alterações moleculares e imunológicas associadas com as diferentes fases da progressão do mesmo, e os avanços destes estudos têm sido dificultados por limitações técnicas para as fases pré-invasivas dos tumores (MACCHETTI et al., 2006).

Durante a última década, vários são os trabalhos sobre os mecanismos relacionados à interação entre as células do sistema imunológico e progressão do tumor. Os resultados indicam que a imunidade ao tumor é determinada em grande parte pelo

tipo de resposta imunológica vigente, bem como interações entre hormônios, proteínas e receptores presentes na superfície de células. No entanto, o papel do sistema imune na prevenção do câncer é complexo e parcialmente compreendido (PARODI, 2007).

É amplamente conhecido que a dieta tem um papel fundamental na etiologia do câncer (PARODI, 2007) e que a amamentação se associa a menor incidência de câncer de mama. Neste sentido, estudos têm tentado elucidar os efeitos da lactação sobre o câncer de mama (FRANÇA-BOTELHO et al., 2012; FRANÇA et al., 2013). Há evidências de que o leite humano pode conferir benefícios a longo prazo (DAVIS et al., 2001; KENT, 2007) e um número crescente de trabalhos tem indicado que o aleitamento materno oferece proteção contra o câncer de ovário e o de mama. No entanto, os efeitos da amamentação sobre o risco de câncer de mama têm sido difíceis de estudar devido à alta correlação com a paridade (BARNETT et al., 2008; ALSAKER et al., 2011). Fatores reprodutivos podem induzir alterações permanentes no epitélio da glândula mamária ou estroma circundante (RUSSO et al., 2005; 2008).

Embora os mecanismos não tenham sido completamente elucidados, a hipótese de reduzir o risco de câncer da mama através da amamentação parece ocorrer devido à diferenciação de tecido mamário ou pela redução do número de ciclos ovulatórios de vida (YANG et al., 2008). É mais provável que as alterações do tecido podem tornar a mama mais ou menos sensíveis a fatores carcinogênicos (RUSSO et al., 2005).

As glândulas mamárias lactantes são parte integrante do sistema imune de mucosa, e os anticorpos e células presentes no leite refletem a estimulação antigênica do Tecido Linfóide Associado a Mucosa (MALT), tanto do intestino como das vias respiratórias. A literatura tem relatado que anticorpos e células do leite humano têm especificidade para uma variedade de antígenos oriundos de patógenos intestinais e respiratórios (GOLDMAN, 2002)

O leite materno pode ser classificado a partir do início da lactação, em colostro (1-7 dias após o parto), leite de transição (7-15 dias pós-parto) e leite maduro (15 dias pós-parto) (GOLDMAN, 1993; ALMEIDA e NOVAK, 2004; CARVALHO E TAMEZ, 2002; FRANÇA et al, 2010; FRANÇA et al, 2013).

O colostro difere da maioria das secreções por conter leucócitos viáveis (10^9 cel/ml) durante os primeiros dias de lactação (ISLAM et al.,2006), com quantidades e atividades comparáveis aos leucócitos do sangue (HONORIO-FRANÇA et al., 1997).

Temos que considerar componentes de defesa presentes na secreção que podem estar associados à atividade de proteção, tais como, lactoferrina, complexos análogos (receptores), ácidos graxos (lipídeos), mucinas (BRANDTZAEG, 2003; 2010), citocinas e quimiocinas (KVERKA et a*l*., 2007; MEKI et al., 2003; LÖNNERDAL, 2003; GAROFALO, 2010), lisozimas (LÖNNERDAL, 2003), probióticos, anticorpos como SIgA (imunoglobilina A secretória) (NEWBURG, 2005), fatores antioxidantes (FRIEL et al., 2008), entre outros componentes produzidos pelo sistema imune materno, além de vários hormônios, como a melatonina (LÖNNERDAL, 2000; MIRALLES et al., 2006; FAGUNDES et al., 2012).

2.5 Imunoglobulina A secretória (SIgA), quimiocinas e Meltonina

O leite materno é particularmente rico em anticorpos SIgA (NEWBURG, 2005), que desempenham um papel protetor contra várias doenças. Esses anticorpos neutralizam os patógenos e, simultaneamente, limitam os efeitos prejudiciais da inflamação tecidual causada por outros tipos de anticorpos (JACKSON; NAZAR, 2006).

A atividade biológica da SIgA é importante porque essa proteína é a classe de anticorpos primários no leite materno e porque sua atividade pode estar mais ligada com o efeito da amamentação na redução da incidência de câncer de mama (HONORIO-FRANÇA et al., 1997; FRANÇA et al., 2011b; HONORIO-FRANÇA et al., 2016).

Estudos sobre a natureza molecular do receptor para a região Fc de SIgA presente na superfície dos fagócitos mostraram que este receptor é constituído por uma proteína heterogênea, glicosilada, com tamanhos que varia de 55 a 100 KDa (CHEVALIER et al., 1989; VIDARSSON et al., 1998; HONORIO-FRANÇA et al., 2001; MONTEIRO et al., 2003). O FcαR em fagócitos do colostro foi identificado com peso molecular (55-75 KDa) similar à de fagócitos do sangue (MONTEIRO, 2003). A interação da SIgA e seu receptor FcαR (CD89) ativa os mecanismos intracelulares resultando no aumento da fagocitose, liberação de ânion superóxido e da atividade microbicida (HONORIO-FRANÇA et al., 1997; FRANÇA et al., 2011b), bem como promove citotoxicidade em células tumorais, podendo ser um candidato para imunoterapia no combate às várias doenças malignas (OTTEN et al.,2005).

As quimiocinas desempenham papel crucial na movimentação das células mononucleares pelo corpo e sua migração para os tecidos, contribuindo para a resposta imune adaptativa e patogênese de várias doenças. Os receptores de quimiocinas são expressos em leucócitos, células dendríticas e células de Langerhans. A maior variedade de receptores é observada em Linfócitos T e sua expressão pode definir o padrão migratório e até mesmo facilitar a identificação de certos subtipos de Linfócitos T. A ligação quimiocina-receptor inicia uma complexa cascata de sinalização que gera respostas quimiotáticas, degranulação, liberação de radicais livres de oxigênio e alteração na afinidade das integrinas presentes na superfície celular (CYSTER, 1999). A expressão de receptores de quimiocinas pode servir como um marcador para maturação e diferenciação de linfócitos (BORISH; STEINKE, 2003).

A proteína quimiotática de monócitos-1 (CCL2/MCP-1), é produzida predominantemente por macrófagos e células endoteliais e é conhecida pelo recrutamento de monócitos, macrófagos, células T de memória, e células dendríticas a sítios de inflamação e tumores (AGARWAL et al, 2011). No tumor está associada à atração de macrófagos associados a tumores (M2) o que leva a um aumento da angiogênese tumoral, sustentação da proliferação, permitindo a metástase das células tumorais. Evidências clínicas, sugerem que a liberação de quimiocinas, como a MCP-1, medeia a migração de monócitos da circulação sanguínea para tumores mamários, onde se tornam macrófagos ativos, contribuindo assim para a progressão do câncer (HULTGREN et al., 2017)

A CXCL8/IL-8 (interleucina-8) é secretada por vários tipos de células, incluindo monócitos, linfócitos T ativados, neutrófilos, eosinófilos, fibroblastos, células sinoviais, adipócitos, células endoteliais e queratinócitos (STEINER et al., 2002), e seu principal papel é no recrutamento de neutrófilos para os sítios inflamatórios e ativação dessas células (KOBAYASHI, 2008) é acompanhada por atividade quimioatrativa de basófilos e células T, e por potente ação pró-angiogênica (BAGGIOLINI et al., 1993).

A quimiocina CXCL9/MIG é uma pequena molécula induzida por interferon gamma (IFN-γ) por células mononucleares estimuladas (FERREIRA et al., 2008), é secretada por células da glia in vivo e por endotélio e granulócitos mediante a incorporação de IFN-γ e ligação ao receptor toll-like (TLR) (GASPERINI et al, 1999). Age também sobre as células T $CD4^+$ Th1 ativadas, células $TCD8^+$ e células NK (MÜLLER et al., 2010).

A IP-10 (CXCL10) é uma proteína induzida por interferon-gama com capacidade antimicrobian,a conhecida por atrair os monócitos, macrófagos, células T ativadas, células NK e células dendríticas. Células, tais como monócitos, neutrófilos, fibroblastos, e as células endoteliais podem produzir IFN-γ necessário para induzir CXCL10. Esta quimiocina é predominantemente sintetizada no fígado e no rim e pode ser ligada à inflamação deste órgão. É membro da família CXC das quimiocinas, que tem o papel de atrair os linfócitos e neutrófilos (AGARWAL et al, 2011). MIG e IP-10 são muito semelhantes e foram relatados por compartilhar atividades biológicas semelhantes, incluindo a capacidade de induzir quimiotaxia de células T, células NK, inibição de hematopoese das células $CD34^+$ derivadas da medula óssea e inibição de angiogênese (SARRIS et al.., 1993; KAI et al.,2002)

Estudos tem comprovado que o aumento da expressão de MIG em camundongos imunocompetentes, fez com que tais camundongos desenvolvessem tumores de pulmão menores e com menos metástases, além de que concentrações aumentadas de MIG e IP-10 no local do tumor direcionou para inibição dos mesmos, aumentando células $TCD4^+$ (linfóticos T helper) e $TCD8^+$ (linfócitos T citotóxicos) no local (ADDISON et., 2000; WALSER et al., 2007).

A quimiocina RANTES, também chamada CCL5, é uma quimiocina que em resposta pró-inflamatória recruta células T, eosinófilos e basófilos para os sítios de inflamação. É considerada um regulador chave para eosinófilos nas vias aéreas e linfócitos e ajuda a induzir a proliferação e ativação de células Natural Killer (ULLER et al., 2010).

Dentre os hormônios encontrados no leite materno, destacam-se a presença de melatonina (HONORIO-FRANÇA et al., 2013; MORCELI et al., 2013; SILVA et al., 2013). A melatonina é sintetizada principalmente pela glândula pineal (CLAUSTRAT et al., 2005). Possui papel importante no controle do ritmo circadiano, reprodução, sono-vigília, está ligada diretamente na regulação de diversos eixos neuroendócrinos (REITER et al.,2013) e apresenta também outras ações protetoras (FRANÇA-BOTELHO et al., 2011; FRANÇA- HONORIO- et al., 2013). Estudos demonstram que a melatonina pode aumentar a imunidade inata e a adquirida e estimular principalmente leucócitos, o que representa um importante mecanismo de proteção para doenças (FRANÇA-BOTELHO et al., 2011; HONORIO-FRANÇA et al., 2013; MORCELI et al., 2013). A melatonina

mostra uma versatilidade funcional notável que exibe propriedades imunomoduladoras, oncostáticas, antioxidantes e antienvelhecimento (CARRILLO-VICO et al., 2004)

A produção de melatonina, por células do sistema imunológico, ocorre por ativação de agentes pró-inflamatórios por exemplo, por citocinas. A melatonina produzida por células imunocompetentes aumenta a capacidade fagocítica dos macrófagos e linfócitos e induz a síntese de interleucina-2 (IL-2), possuindo ação autócrina e parácrina (CARRILLO-VICO et al., 2004).

A ação da melatonina direta contra radicais livres tem sido cada vez mais estudada. O seu papel indireto como antioxidante foi testado e o seu efeito foi altamente eficaz na redução do estresse oxidativo no organismo, quando comparada com os antioxidantes mais conhecidos como as vitaminas C e E. A melatonina e vários dos seus metabólitos tem vários aspectos positivos que a tornam eficiente no combate de radicais livres, eles atravessam facilmente a barreira hematoencefalica e placenta, além de todos os órgãos maternos o que leva a uma maior proteção da placenta e do feto. Outros aspectos positivos da melatonina é que ela pode ser produzida em outros compartimentos, extra pineais (fora da glândula pineal) em vertebrados, como por exemplo nas mitocôndrias, porém não de forma circadiana, mas sim como antioxidante combatendo a geração de radicais livres (REITER et al.,2013; 2014).

A concentração de melatonina no colostro humano e leite maduro repercute a concentração deste hormônio da circulação sanguínea, porém células imunocompetentes do colostro passam a produzir melatonina após estímulos de injúrias, como os causados por bactérias, durante infecções com *Escherichia coli*. Sendo que essa produção de melatonina é dependente do estímulo, estudos comprovaram que linfócitos e macrófagos peritoneais de ratos, e fagócitos de colostro humano produzem melatonina em resposta à ativação, e essa produção de melatonina no local ativa linfócitos e macrófagos para produção de IL-12, IL-6, IFN-γ, o que aumenta a produção de linfócitos T, a apresentação de antígenos e a atividade fagocítica de macrófagos, aumentando assim o aspecto inflamatório (GARCIA-MAURINO et al., 1999; PONTES et al., 2006; 2007).

Células imunocompetentes da mama permanecem altamente permeáveis após o parto, o que torna esse tipo celular bom para estudos de vias de sinalização, quando coletadas de forma não invasiva, apontando que essas células desempenham um papel importante na proteção do recém-nascido (PONTES et al., 2006).

Com concentrações semelhantes ao do plasma sanguíneo a melatonina pode exercer um efeito anti-inflamatório, regulando a migração celular ao inibir o rolamento e adesão de leucócitos (LOTUFO et al., 2006).

Estudos que avaliaram a correlação entre produção de melatonina e produção de TNF-α (fator de necrose tumoral alfa), encontraram uma correlação negativa entre essas duas substâncias uma vez que a citocina pró-inflamatória TNF-α em níveis aumentados inibe a produção de melatonina. Porém, células do sistema imunológico podem promover a produção de melatonina de forma autócrina. Esse efeito então deve ser caracterizado dependente do local de produção e quantidade produzida, e não apenas pelo caráter anti ou pró- inflamatório (PONTES et al., 2006; 2007).

A melatonina exerce efeito antiproliferativo em células MCF-7 (linha celular de adenocarcinoma de mama humano) de câncer de mama, dependente da dose fisiológica, além de reduzir as taxas de propriedades invasivas e metastáticas desse tipo celular (COS et al., 1998). Estudos comprovaram que a melatonina diminuiu a proliferação celular e aumentou a expressão das proteínas p53 e p21 em células MCF-7, inibindo a proliferação e induzindo a apoptose. A proteína p53 é um importante gene supressor de tumor, p53 está envolvido na regulação do ciclo celular (COS et al., 2002). A melatonina, via ativação do receptor de melatonina 1 (MT1) (ROGELSPERGER et al., 2009), está associada à supressão do crescimento e desenvolvimento do câncer de mama, através da regulação de fatores de crescimento, regulação de expressão gênica, pela inibição da invasão de células tumorais e metástase e pela regulação do desenvolvimento da glândula mamária (HILL et al., 2009).

Existe a produção de melatonina por outras células e órgãos, que não a glândula pineal, como retina, timo, cérebro, intestino, medula óssea, ovário, testículos, placenta, pele, e linfócitos. Altas concentrações de melatonina foram encontradas em queratinócitos da pele, o que sugere que a produção de melatonina fora da glândula pineal não está relacionada ao ritmo circadiano claro/ escuro, mas sim como agente antioxidante e anti-inflamatório, como mecanismo de proteção do estresse oxidativo. Essa produção de melatonina em resposta ao estresse oxidativo ocorre em todos os seres vivos, como plantas, seres unicelulares, animais e o homem. Várias doenças como Alzheimer, câncer, doença cardíaca, mostram níveis baixos de melatonina nos pacientes, porém como essas doenças produzem muitas espécies reativas de oxigênio, ainda não se

sabe se ocorre uma diminuição da produção da melatonina ou se a melatonina interage com as espécies reativas e é consumida na reação (TAN et al., 2007).

Estudo de HARA e colaboradores (2013) demonstrou que a melatonina adsorvida à microesfera de PEG foi capaz de aumentar a atividade funcional de fagócitos do colostro e que este sistema modificado de liberação do hormônio pode representar uma alternativa no tratamento de doenças.

2.6 Polietilenoglicol (PEG)

O polietilenoglicol é um polímero, muito utilizado pela área farmacêutica, pois são importantes para modulação, prolongamento da ação da droga e não apresenta efeitos tóxicos (HARRIS; CHESS, 2003). Além disso, possui propriedades que podem melhorar a biocompatibilidade dos materiais e fluidos biológicos (HUANG et al.,1999; PARK; KIM, 2004; RIBEIRO et al.,2018).

Para a aplicação na terapia humana de produtos biofarmacêuticos é utilizado a técnica de "PEGylation", onde ocorre o acoplamento covalente de polietilenoglicol às cadeias dos fármacos. Este processo protege a formulação farmacêutica da sua superfície para a periferia. Assim, a estabilidade destes conjugados contra proteases aumenta, reduz a imunogenicidade e diminuiu a excreção renal. Com isso, a técnica assegura uma meia-vida prolongada, reduz os efeitos secundários e, finalmente, aumenta a eficiência da terapia (DAVIS, 2002; ABUCHOWSKI et al., 1977).

Estudos visando reduzir efeitos adversos de medicamentos têm sido desenvolvidos como novos sistemas terapêuticos, conhecidos como sistemas de liberação modificada (VERMA e GARG, 2001; GIL et al., 2006; BATISTA et al., 2007; KREUTER, 2007; GRABOVAC et al., 2008). A melhoria das propriedades terapêuticas de compostos bioativos por meio da incorporação em microcarreadores consiste em uma importante estratégia na obtenção de terapias. Visto que são eliminadas alterações cíclicas da concentração, há disponibilidade biológica do composto, bem como a redução na toxicidade, no número de doses administradas e supressão de reações adversas (ALAGUSUNDARAM et al., 2009).

Conjugados PEG-droga estão sendo estudados como possíveis sistemas de liberação modificada para uma variedade de moléculas e drogas (GREENWALD et al.,

2003; PARK et al., 2005; YU et al., 2005; VERONESE e PASUT, 2005; SALMASO et al., 2005; HEYES et al., 2006; RODRIGUES, 2006; SCOTT et al., 2010). Essa combinação apresenta inúmeras vantagens como permanência prolongada no organismo, decréscimo da degradação por enzimas metabólicas e redução ou eliminação da imunogenicidade de proteínas (YU et al., 2005).

Vários estudos mostram que a associação de microesferas de PEG a moléculas, hormônios ou proteínas, apresenta capacidade imunomoduladora sobre os fagócitos, tanto do sangue como de colostro, e sugerem que a adsorção destes compostos a microesferas de PEG tem efeitos imunoestimulatórios e pode ser considerado um importante material, com potencial para futuras aplicações terapêuticas em doenças infecciosas ou em tumores (SCHERER et al., 2011; FAGUNDES et al., 2012; REINAQUE et al., 2012; HARA et al., 2013; GUIMARÃES et al., 2013; FRANÇA et al., 2014).

Considerando-se que o tecido mamário está em contato constante e direto com os componentes imunes solúveis e celulares do leite materno. E os inúmeros constituintes imunológicos do leite materno, entre estes altas concentrações de SIgA, melatonina e células mononucleares, é possível que interações destes componentes, de forma direta ou por sistemas de liberação modificada, com fatores presentes em células tumorais possam constituir uma alternativa para a imunoterapia de tumores.

3. OBJETIVOS

3.1 Objetivo Geral

O objetivo deste trabalho foi avaliar a ação de agentes imunomoduladores associados a sistemas de liberação modificada sobre células mononucleares de colostro em co-cultura com células de tumor mama.

3.2 Objetivos Específicos

Imunomodulador: SIgA adsorvido ou não a microesfera de PEG:

- Analisar a imunofenotipagem e os percentuais de células $CD3^+$, $CD4^+$, $CD8^+$, $CD14^+$ do colostro em presença das células tumorais;

-Analisar a concentração de quimiocinas (IL-8, MIG, RANTES, IP-10 e MCP-1) no sobrenadante da cultura de células MN, MCF-7 e co-cultura (células MN e MCF-7);

- Determinar viabilidade de células MN do colostro, células MCF-7 e co-cultura (células MN e MCF-7);

- Verificar a liberação de cálcio intracelular pelas células MN do colostro, células MCF-7 e co-cultura (células MN e MCF-7);

- Avaliar os índices de apoptose de células MN do colostro, células MCF-7 e co-cultura (células MN e MCF-7);

Imunomodulador: melatonina adsorvido ou não a microesfera de PEG:

- Avaliar em células MN do colostro, células MCF-7 e co-cultura (células MN e MCF-7) a liberação do ânion superóxido em diferentes fases do dia;

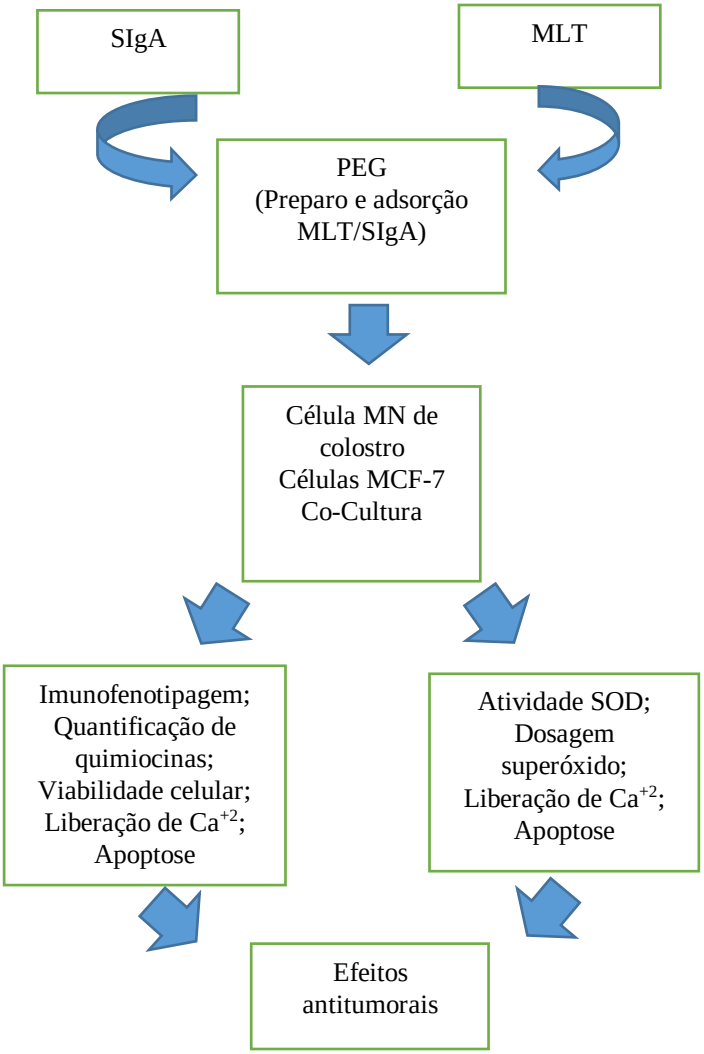
- Analisar em cultura de células MN do colostro, células MCF-7 e co-cultura (células MN e MCF-7) a concentração da enzima superóxido dismutase (SOD) em diferentes fases do dia;

-Verificar em células MN do colostro, células MCF-7 e co-cultura (células MN e MCF-7) a liberação de cálcio intracelular em diferentes fases do dia;

-Quantificar os índices de apoptose de células MN do colostro, células MCF-7 e co-cultura (células MN e MCF-7);

4. METODOLOGIA

4.1 Delineamento experimental



4.2 Local da pesquisa

As amostras de colostro humano foram coletadas no Hospital Municipal de Barra do Garças, sendo alíquotadas, congeladas e enviadas ao LABIMUNOBIO InBio/UFMS (Universidade Federal de Mato Grosso do Sul) e Laboratório Materno Infantil/ UFMT (Universidade Federal de Mato Grosso), para o processamento. Este projeto foi aprovado com apoio financeiro do Edital Universal CNPq - 447218/2014-0; Nº: 308600/2015-0).

4.3 Sujeitos

As amostras de colostro foram obtidas de mães saudáveis, com idade entre 18 e 40 anos. As mães foram consultadas sobre a disponibilidade em doar uma amostra de colostro (aproximadamente 8 ml). A coleta foi efetuada através de ordenha manual. Para avaliar os efeitos da SIgA foram coletadas 30 amostras de colostro, sempre no período da manhã e no intervalo entre duas mamadas, no período correspondente as primeiras 48 a 72 horas após o parto.

Para avaliar os efeitos da melatonina a coleta do colostro foi realizada seguindo o protocolo desenvolvido por França et al. (2010). Foram coletadas 60 amostras de leite materno em dois períodos, sendo uma no período diurno e outra no período noturno) totalizando 120 amostras, também no intervalo entre duas mamadas, no período correspondente as primeiras 48 a 72 horas após o parto. Todas as mães tiveram seus bebês a termo saudáveis por meio de parto cirúrgico.

4.4 Linhagem de células tumorais e cultura de células

Para os ensaios biológicos com os imunomoduladores foram utilizadas linhagens celulares ATCC (American Type Culture Collection, USA) de adenocarcinoma de mama (MCF-7).

A linhagem foi cultivada e congelada em nitrogênio líquido para estocagem. Posteriormente foram cultivadas em uma mistura 1:1 DMEM e Ham's F10 (Sigma), acrescido de HEPES, penicilina, estreptomicina, bicarbonato de sódio e soro fetal bovino (SFB, Cultilab). As células foram cultivadas em garrafas e mantidas a 5% de CO_2 e a

temperatura de 37ºC até a formação de monocamada celular, depois as garrafas de células foram submetidas a tripsinização, para isso, as células foram lavadas com 5 mL RPMI, e as linhagens serão submetidas à 1 mL de tripsina 0,05% EDTA produzidas a partir de uma solução 10 vezes concentrada (In vitrogen, EUA) até o desprendimento das células do fundo das garrafas. A seguir, as células foram homogeneizadas com volumes variados do meio de cultura acrescido de 10% de soro fetal bovino para a neutralização da tripsina. O volume da suspensão celular obtido em uma garrafa foi transferido para outras duas garrafas, de modo a obter quantidade celular adequada para os experimentos. As células foram tratadas com diferentes imunomoduladores (SIgA e MLT) adsorvidos ou não a microesfera de PEG. Como controle negativo, a linhagem não foi submetida ao tratamento (células não tratadas).

4.5 Obtenção de fagócitos do colostro

Foram coletadas amostras de colostro com volume aproximado de 8ml. O colostro foi centrifugado por 10 minutos a 160 xg sob refrigeração a 4ºC. Após centrifugação houve a formação de 3 fases diferentes: pellet celular, uma fase aquosa intermediária e um sobrenadante contendo lipídios. A camada de gordura superior foi descartada, o sobrenadante aquoso armazenado a -80°C para a obtenção de SIgA purificado.

As células foram ressuspendidas em meio de cultura 199 (Gibco), e separadas em gradiente de densidade com Ficoll-Paque (Pharmacia) por 40 minutos a 160 G, sob temperatura de 4ºC. A seguir foram contadas em câmara de Newbauer, e as concentrações celulares ajustadas para 2 x 10^6 células/ml. As células mononucleares foram utilizadas nos ensaios de imunofenotipagem, liberação de ânion superóxido, de atividade microbicida, liberação de cálcio intracelular, proliferação e apoptose.

4.6 Purificação de SIgA de colostro humano

O SIgA de colostro humano foi purificado a partir de um “pool” de sobrenadante colostro deslipidado por cromatografia de afinidade em Sepharose-4B ativada com brometo de cianogênio (CNBr-Sepharose-4B - Sigma, ST Loius, EUA) ligada com cadeia α anti-humano de ovelha como proposto por March et al. Para garantir a depleção de SIgA, as frações eluídas da coluna de cromatografia de afinidade foram

reunidas e passadas cinco vezes pela mesma coluna. O SIgA ligado foi eluída da coluna com tampão 6N de glicina - HCL, pH 2,8. Os preparados purificados foram restaurados para o volume inicial. A concentração de SIgA foi determinada por imunodifusão radial simples com um soro de cadeia α anti-humana de ovelha em placas de agarose. A concentração total de proteína estava disponível pelo método de Lowry. A preparação de SIgA purificada também foi testada por imunoeletroforese com antisoros de cabra anti-γ humano e cadeia μ. Tanto a IgG quanto a IgM foram indetectáveis na preparação. O SIgA purificado foi 4,0 g / L ajustado para uma concentração de 100 ng / mL. As alíquotas foram armazenadas a -80 ° C e posteriormente utilizadas para os experimentos.

4.7 Preparação de microesferas de polietilenoglicol (PEG) e incubação com agentes imunomoduladores

As microesferas foram obtidas a partir de polietilenoglicol 6000, usando o método descrito por (SCHERER et al., 2011). Em que 20 g de PEG 6000 foi suspenso em 100 mL de tampão salina fosfato (PBS), foi diluída com uma solução de 2 g de sulfato de sódio em PBS e incubadas a 37°C durante 45 min. Após a incubação, as microesferas de PEG foram diluídas 3:1 em PBS e lavadas duas vezes em PBS (500 xg, 5 min). As microesferas de PEG foram ressuspendidas em PBS. A formação de microesferas foi induzida termicamente, submetendo a solução a 95°C durante 5 minutos. Para a adsorção, as suspensões de microsferas a PEG, em PBS, foram incubadas com os imunomoduladores - MLT (Sigma, concentração de 100 ng/mL), ou sIgA (do próprio colostro - concentração de 100 ng/mL) a 37 ° C durante 30 minutos. Após este período, as microesferas de PEG com os imunomoduladores adsorvidos foram lavadas duas vezes em PBS (500 x g, 5 min). O carregamento eficiente da adsorção das microesferas de PEG com MLT ou SIgA foram verificados por microscopia de fluorescência através da marcação com uma solução de Dylight-488 (Pierce Biotechnology, Rockford, EUA, 10 μg mL-1) em dimetilformamida a 100:1 proporção molar de PEG: Dylight (HARA et al, 2013).

4.8 Tratamento de células MN, MCF-7 e co-cultura com SIgA e MLT

Para avaliar os fenótipos de células, a viabilidade celular (teste de permeabilidade ao iodeto de propídio), a indução de apoptose (coloração de anexina) e a liberação de cálcio intracelular (Fluo-3 fluorescente) as células MN, MCF-7 e co-cultura foram pré-incubadas durante 24h com ou sem 50 µL de SIgA (100 ng / mL), 50 µL de microesferas de polietilenoglicol (PEG) ou 50 µL de SIgA adsorvido em microesferas de PEG (PEG-SIgA) (concentração final de 100 ng / mL).

Já para a liberação de superóxido, a superóxido dismutase em sobrenadante de cultura, a liberação intracelular de cálcio e a indução de apoptose em células MN, MCF-7 e co-cultura (fases diurno e noturno) foram pré-incubadas durante 24 h com ou sem 50 µL de melatonina (100 ng / mL de concentração final - Honorio -França et al., 2013), 50 µL de microesferas de polietilenoglicol (PEG) ou 50 µL de melatonina adsorvido em microesferas de PEG (PEG-MLT) (concentração final de 100 ng / mL).

Em seguida, as células foram diluídas em meio RPMI 1640 suplementado com 10% de soro bovino fetal (FBS) (Sigma, St. Louis, MO, EUA), penicilina (20 U / mL) e estreptomicina (20 µg / mL) (Sigma, St. Louis, MO, EUA) a 37°C durante 24 h em estufa de CO_2 a 5%.

4.9 Imunofenotipagem de células incubadas com SIgA

Após o tratamento da cultura, as células do colostro em co-cultura ou não foram coradas com 5 µL de anti-CD3 PerCP; anti-CD4FITC, anti-CD8PE e anti-CD14FITC durante 30 min à temperatura ambiente. As células foram lavadas e ressuspensas em solução salina tamponada com fosfato (PBS) contendo albumina de soro bovino (BSA-Sigma, ST Louis, EUA; 5mg / mL) para análises de citometria de fluxo. Controle isotípico (IgG1-FITC, IgG1-PE tanto de BD Biosciences). Um mínimo de 10.000 células foi analisada por tamanho (FSC) e granularidade (SSC) com um citômetro de fluxo (FACSCalibur, BD Biosciences, EUA). Os dados foram analisados usando o software Flowjo 7.2.5.4.4.5.

4.10 Quantificação de quimiocinas no sobrenadante das culturas incubados ou não com SIgA

Quimiocinas interleucina-8 (CXCL8), RANTES (CCL5), MIG (CXCL0), MCP-1 (CCL2) e IP-10 (CXCL10) no sobrenadante de culturas de células MN, células MCF-7 e co-cultura (células MN e MCF-7) foram medidas por um kit comercial cytometric Beads Array (CBA, BD Biosciences, EUA) de acordo com a procedimentos do fabricante. A aquisição de amostras foi realizada dou um citômetro de fluxo (FACSCalibur, BD Biosciences, EUA), e a análise dos dados pelo software CellQuest (BD Biosciences, EUA) FCAP Array 3.0 (BD).

4.11 Viabilidade celular das culturas tratada com SIgA

Após 24 h de tratamento com SIgA, PEG e PEG-SIgA, o meio de cultura foi removido e as células (células MN e / ou células MCF-7) foram lavadas duas vezes com solução salina tamponada com fosfato (PBS). O teste de viabilidade foi realizado utilizando o ensaio de fluorescência de iodeto de propídio. As células foram então coradas com 10 μL de iodeto de propídio (PI, 1 mg / mL), Triton X-100 (5,5%) e EDTA (110 mM) e incubadas durante 10 min à temperatura ambiente. As células não tratadas foram usadas como controles. A fluorescência das células foi analisada por citometria de fluxo (FACSCalibur, BD, San Jose, EUA). A porcentagem de células viáveis foi inversamente proporcional à intensidade de fluorescência média geométrica de PI.

4.12 Liberação de Cálcio Intracelular pelos fagócitos do colostro tratados com SIgA e MLT

Para verificar se os mecanismos intracelulares de atividade dos fagócitos ocorrem na dependência de liberação de cálcio intracelular foi utilizado o corante fluorescente Fluo-3 (Sigma - Fagundes et al., 2012). Suspensões de células de colostro foram pré-incubadas com os imunomoduladores, misturadas a 37ºC por 30 min, sob agitação contínua. A seguir foram centrifugadas duas vezes (1500 rpm, 10 min, 4ºC) ressuspendidas em PBS contendo BSA (5 mg/mL) e incubadas com 5 μL de Fluo-3 (1 mg / mL) por 30 min a 37ºC. Após a incubação, as células foram lavadas duas vezes em PBS contendo BSA (5 mg/mL; 1500 rpm, 10 min, 4 ºC) e analisadas por Citometria de Fluxo FACSCalibur (BD San Jose EUA). O Fluo-3 foi detectado utilizando-se filtro na

faixa de 530/30nm. A quantificação da liberação de cálcio intracelular foi expressa como a média geométrica da intensidade de fluorescência do Fluo-3.

4.13 Ensaio de apoptose das culturas de células tratados com SIgA e MLT

A coloração de Annexin V foi utilizada para avaliar a apoptose. As células não tratadas foram utilizadas como controles negativos, e as células tratadas com estaurosporina (Sigma, St. Louis, EUA; Pundt et al.,2009 que foram utilizadas para induzir apoptose, foram utilizadas como controles positivos. As células (células MN e / ou MCF- 7 células) foram ressuspensas em 500 μL de tampão de ligação contendo 5 μL de anexina V-FITC (Kit de Detecção de Apoptoses de Anexina V-FITC, AlexisTM, San Diego, EUA) e 5 μL de PI e depois incubados durante 10 min à temperatura ambiente. A fluorescência das células foi analisada por citometria de fluxo (sistema FACSCalibur, BD, San Jose, EUA). Os dados obtidos foram analisados usando o software CellQuest. As células foram classificadas da seguinte forma: células viáveis (anexina - / PI -), células apoptótica precoce (anexina + / PI -), células apoptóticas tardias (anexina + / PI +) e células necróticas (anexina - / PI +).

4.14 Atividade enzimática da superóxido dismutase (SOD) (CuZn-SOD - E.C.1.15.1.1) das culturas de células tratados com MLT

O princípio do método de determinação da atividade enzimática do superóxido dismutase consiste na ação da enzima catalisar por meio de dismutação o superóxido de oxigênio e peróxido de hidrogênio. A enzima SOD presente na amostra inibe a redução do azul de nitrotetrazólio (NBT) pelo ânion superóxido. O ânion superóxido é gerado a partir da auto-oxidação da hidroxilamina em pH alcalino.

A atividade enzimática da CuZn-SOD foi medida no sobrenadante das culturas de MN, MCF-7 e co-cultura MN-MCF-7 dos quais foram pipetados 0,5 mL de cada amostra e colocados em tudos de ensaio (vidro), conforme protocolos elaborados para os grupos.

Junto às amostras foram adicionados 0,5 mL de mistura clorofórmio-etanol (1:1), 0,5 mL da mistura reativa de NBT em conjunto com ácido etilenodiamino tetra-acético (EDTA) (1:1.5) e 2,0 mL de tampão carbonato com hidroxilamina, perfazendo ao final

uma solução com volume de 3,5 mL. A mistura reativa foi utilizada como branco para calibrar o aparelho e para a análise do padrão foram utilizados 0,5 mL de mistura hidro-alcoólica (1:4), 0,5mL de mistura clorofórmio-etanol (1:1), 0,5 mL da mistura reativa de NBT e EDTA (1:1.5) e 2,0 mL de tampão carbonato com hidroxilamina.

Todas as reações foram incubadas em temperatura ambiente por 15 minutos e após esse período a reação foi paralisada em gelo. A análise foi realizada a partir da inibição da redução do NBT (Sigma, St Louis, MO, USA) pelo ânion superóxido e lidas com espectrofotômetro à 560 nm (CROUCH et al., 1981). A enzima catalisa o ânion superóxido que é gerado pela adição de hidroxilamina em pH alcalino.

A quantidade de enzima foi determinada a partir da seguinte fórmula:

$$SOD = \frac{(Ab.Padrão - Ab.Amostra)}{Ab.Padrão}\ x\ 100 = \%\ de\ redução\ do \frac{NBT}{CuZn - SOD}$$

O resultado foi expresso por unidades internacionais (UI) de CuZn-SOD (CROUCH et al., 1981).

4.15 Dosagem de ânion superóxido das culturas de células tratados com MLT

A atividade dos fagócitos do colostro incubados com a MLT adsorvidos ou não a microesfera de PEG, na presença de células tumorais foi verificada através da liberação de ânion superóxido, utilizando-se o cromógeno Ferricitocromo C, segundo o método de Pick & Mizel (1981) e adaptado por Honorio-França e colaboradores (1997). Em presença do ânion superóxido o ferricitocromo C sofre oxidação passando a ferrocitocromo C, sendo esta mudança colorimétrica detectável em espectrofotômentro com filtro de 550 nm.

A leitura foi feita em espectrofotômetro para placa com filtro de 550 nm. A concentração do ânion superóxido foi calculada através da seguinte relação:

Concentração O_2^- (nmol) =DO/6.3 x 100

4.16 Análise estatística

Para análise estatística de todos os ensaios foi utilizado o teste de Análise de Variância (ANOVA) seguida de testes de comparações múltiplas de Bonferroni, no software Bioestat 5.0. As estatísticas foram consideradas significativas quando o valor de $p < 0,05$.

4.17 Aspectos Éticos

O presente estudo foi submetido ao CEP e aprovado com Número CAAE 45102815.3000.5587, Número de parecer 1.064.829 e data da relatoria 15/05/2015 (Anexo 1). As considerações éticas foram baseadas no uso do material biológico para fins científicos, com sigilo da identidade da nutriz, livre de coação ou conflito de interesses da instituição ou de pessoas envolvidas no projeto. As coletas respeitaram os protocolos técnicos dos serviços envolvidos. As nutrizes foram previamente informadas e o material somente foi coletado ou utilizado sob expresso consentimento em formulário específico (Termo de Consentimento Livre e Esclarecido - TCLE), conforme resolução 466/12 do Conselho Nacional de ética em Pesquisa (CONEP) (Apêndice).

5. RESULTADOS E DISCUSSÃO

5.1 Artigo 1: Publicado no International Journal of Advanced Engineering Research and Science (IJAERS)

Melatonin bioengineered: A New Possible Strategy for Treatment of Breast Cancer.

Objetivos: O objetivo desta revisão foi detalhar a influência dos componentes solúveis e celulares presentes no colostro humano, como o hormônio MLT, e como eles podem ser responsáveis por mecanismos que reforçam a hipótese de que a amamentação reduz o risco de câncer de mama.

5.2 Artigo 2: Submetido na revista Chronobiology International.

Time-dependent immune effects by melatonin on colostral cells in co-cultured with human breast cancer cells.

Objetivos: O objetivo deste estudo foi investigar o efeito da melatonina adsorvida em microesferas de PEG no estresse oxidativo e apoptose em células MN de colostro humano co-cultivadas com linhagens tumorais de câncer de mama (MCF-7).

5.3 Artigo 3: Em elaboração

Modulation chemokines release in culture of colostral mononuclear cells and human breast cancer cells influenced by nanodoses of secretory IgA adsorbed to PEG microspheres.

Objetivos: O objetivo deste estudo foi investigar o efeito das microesferas de PEG com nanodoses de SIgA adsorvido nas células mononucleares de colostro humanas co-cultivadas com linhagens celulares tumorais de câncer de mama (MCF-7).

5.1 Artigo 1; Publicado no International Journal of Advanced Engineering Research and Science (IJAERS)

International Journal of Advanced Engineering Research and Science (IJAERS) *[Vol-5, Issue-10, Oct- 2018]*
https://dx.doi.org/10.22161/ijaers.5.10.2 *ISSN: 2349-6495(P) | 2456-1908(O)*

Melatonin bioengineered: A New Possible Strategy for Treatment of Breast Cancer

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***Abstract*—** *Breast cancer is an important public health problem, with an estimated 3.2 million new cases by the year 2050. Diet plays a key role in the etiology of breast cancer and breastfeeding is associated with a lower incidence of breast cancer. On the other hand, the improvement of the therapeutic properties of bioactive compounds through their incorporation into microcarriers is an important strategy in obtaining new therapies, since cyclical changes in concentration are eliminated; there is biological availability of the compound as well as the reduction in toxicity, number dose and suppression of adverse reactions. Studies using hormones such as melatonin extracted from human milk adsorbed onto polyethylene glycol (PEG) microspheres showed that the controlled release of this compound was able to reduce viability and induce apoptosis in MCF-7 cell lines. Colostrum differs from most of the secretions because it contains viable leukocytes during the first days of lactation with a quantity and activity comparable to blood leukocytes, and has several defense components such as antibodies and hormones, such as melatonin (MLT). This review details the influence of the soluble and cellular components present in human colostrum, such as the MLT hormone, as the modified release systems influence the action of MLT and the possible mechanisms involved that contribute to the hypothesis of reduction of breast cancer in women who breastfed.*

Keywords*— *colostrum, melatonin, polyethylene glycol, breast cancer, bioengineering.

I. INTRODUCTION

Breast cancer cases have increased worldwide, and are directly related with more life expectancy, exposure to risk factors, and habits. Breast cancer is a multifactorial disease with a higher incidence among women, leading to death [1]. Cancer and chronic inflammation are closely linked and the imbalance between reactive oxygen species and antioxidant enzymes favors the emergence of these diseases. Thus the control of the production of reactive species as well as the maintenance of oxidative balance are primordial for the control of tumor progression [2, 3].

Some factors such as healthy lifestyle and breastfeeding are related to the prevention of breast cancer [4]. Human colostrum differs from other secretions by containing large quantities of viable leukocytes comparable to those found in blood acting as anti-inflammatory mediators. It is believed that both soluble and cellular components interact with each other and may be important for antitumor immunity [5,6]. Breast milk is rich in soluble and cellular components, such as

International Journal of Advanced Engineering Research and Science (IJAERS) *[Vol-5, Issue-10, Oct- 2018]*
https://dx.doi.org/10.22161/ijaers.5.10.2 *ISSN: 2349-6495(P) | 2456-1908(O)*

phagocytes, secretory IgA immunoglobulin (SIgA), and hormones, especially melatonin (MLT) [5,7,8].

Melatonin, a hormone produced by the pineal gland, is involved in several physiological processes, including the functional regulation of breast milk. In milk it is related to the anti-inflammatory effects [9] and pro-oxidant and antioxidant effects of paramount importance in the oxidative stress balance as a protection mechanism [10].

Some studies have reported that the bioavailability and biofunctional function of melatonin may be potentiated when associated in modified release systems [11,12]. Among these systems, polyethylene glycol (PEG) microspheres have been considered an important vehicle for the administration of various drugs, natural products and hormones [13,14,15,16,17].

The administration of drugs adsorbed to carrier systems such as PEG microspheres has been an alternative treatment for various diseases [18], including breast cancer. These release systems are promising for the release of the hormone melatonin [11], preventing it from the degradation promoted by the metabolic enzymes increasing their bioavailability in the organism [19]. How much combined with the MLT has been demonstrated its ability to reduce cell viability and induce apoptosis in tumor cell lines from breast cancer [12,11].

The control of the process of carcinogenesis is closely related to the control of apoptosis, since the tumor cell is able to alter this system, favoring its proliferation and promotion [20]. Thus, cancer treatments are directed at inducing increased apoptosis of tumor cells [21, 22], which may be favored by the use of immunomodulatory agents such as MLT, for acting on immunocompetent cells and assisting in tumor eradication.

This review details the influence of the soluble and cellular components present in human colostrum, such as the MLT hormone, and how they may be responsible for mechanisms that reinforce the hypothesis that breastfeeding reduces the risk of breast cancer.

II. BREAST CANCER

Breast cancer is a public health problem, with around 59,700 new cases in Brazil in the year 2018 [23]. The worldwide incidence and mortality of this disease are highly related and it is estimated that by the year 2050 will appear about 3.2 million new cases of breast cancer in the world. Despite technological advances, there are still several mechanisms that must be elucidated in the eradication of this disease that affects the world population [24].

Breast cancer is considered a heterogeneous disease, both morphologically and clinically, and is due to a disordered proliferation of breast tissue cells. About 80% of the types of breast tumors originate in the ductal epithelium, known as invasive ductal carcinoma [25]. Invasive carcinomas are so called because they have high metastatic potential, since carcinomas in situ have low metastatic potential and may arise in both lobes and mammary ducts [26]. During carcinogenesis, genetic mutations are accumulating and the cell phenotype is changing through malignant lesions, evolving into invasive cancer [27].

The structure of the breast is composed of glandular tissues composed of the milk producing glands and the ducts through which milk produced and stromal tissues pass, which are fibrous and fibrous connective tissues. In addition to these tissues the breast is also composed of the tissue of the immune system and lymphatic system [28]. For normal development of breast tissue to occur, there is a need to balance cell proliferation and apoptosis. In tumor growth, there is a reduction in apoptosis and an increase in cell proliferation [27].

The balance between a protective cytotoxic response and a non-protective response can be regulated by the individual's overall immune status [29]. A major challenge for tumor research has been the identification of molecular and immunological changes associated with the different stages of tumor progression, and advances in these studies have been hampered by technical limitations to the pre-invasive stages of tumors [30].

The study with in vitro breast cancer cells began in 1973 from isolated cells from pleural effusion of a 69-year-old woman with metastatic disease [31]. MCF-7 cells are useful for in vitro breast cancer studies by having several particular ideal characteristics of the mammary epithelium, such as the ability to process estrogen in the form of estradiol via estrogen receptors in the cell cytoplasm. This cell line is positive for the estrogen receptor (ER) and for the progesterone receptor and negative for HER2. These cells are very well studied with the immense number of protocols defined which allows researchers to use this cell line for study pathogenesis and in the search for treatment of breast cancer through reliable means in vitro assays [32]. During the last decade, several work on the mechanisms related to the interaction between the cells of the immune system and tumor progression. The results indicate that an immune response to a tumor is determined by the different cell types, such as lymphocytes, NK cells, neutrophils and others, as well as by the interactions between hormones, proteins and receptors present on the cell surface [33].

On the other hand, tumor cells arise from a mutation in DNA (deoxyribonucleic acid) that can be caused by radiation, bacteria, fungi, viruses, chemicals, etc. Although the components of the immune system are present and active, cancer cells can progressively grow

International Journal of Advanced Engineering Research and Science (IJAERS) *[Vol-5, Issue-10, Oct- 2018]*
https://dx.doi.org/10.22161/ijaers.5.10.2 *ISSN: 2349-6495(P) | 2456-1908(O)*

and spread, thus, the body weakened by poor diet, genetic predisposition, advanced age and exposure is the perfect environment for the development of cancer. In addition the cancer cells are very similar to the own cells of the organism, which hampers even more the response of the immune system [28].

Among the mutations, the most important alterations that occur are self-sufficiency in signs of proliferation, insensitivity to growth inhibitory signals, evasion of apoptosis, unlimited replicative potential, sustained angiogenesis and tissue invasion and metastasis [20].

The carcinogenesis process is directly involved with the generation of reactive oxygen species. Oxidative stress participates in all stages of cancer development. At initiation, oxidative species damage DNA by introducing genetic mutations and structural alterations; in the promotion, there is an increase of the initiated cell population, which is proliferation with consequent decrease of apoptosis; already in progression participate in the development of irreversible cancer growth [34].

III. BREASTFEEDING AND THE IMPORTANCE OF BREAST CANCER PREVENTION

Breast cancer is the neoplasm most feared by women, since its occurrence causes great psychological, functional and social impact, acting negatively on issues related to self-image, social interaction and the perception of sexuality. It is considered of great importance in the health care of women, due to the high prevalence, morbidity and mortality [35].

The role of the immune system in cancer prevention is complex and partially understood. It is widely known that diet plays a fundamental role in the etiology of cancer [33] and that breastfeeding is associated with lower incidence of breast cancer. In this sense, studies have tried to elucidate the effects of lactation on breast cancer [36, 37]. There is evidence that human milk may confer long-term benefits and an increasing number of studies have indicated that breastfeeding provides protection against ovarian and breast cancer [38,39].

However, the effects of breastfeeding on the risk of breast cancer have been difficult to study because of the high correlation with parity [40, 41]. Reproductive factors may induce permanent changes in the epithelium of the mammary gland or in the surrounding stromal tissue [42,43]. Although the mechanisms have not been fully elucidated, the hypothesis of reducing the risk of breast cancer through breastfeeding seems to occur because of breast tissue differentiation or reduction in the number of ovulatory cycles [44].

Studies have revealed that the relative risk of having breast cancer reduces about 7,0% for each child born and about 4,3% for every 12 months of breastfeeding. This suggests that breastfeeding duration mothers is crucial to ensure the immunity components acts against the breast cancer [37,45] There is still evidence that breastfeeding protects women who have had their children under 50 years of age [46]. Another study reported that women who breastfed several children had the lowest risk of developing breast cancer, and mothers who breastfed four or more children had a 60 percent reduction in breast cancer risk. The magnitude of the protective effect is directly related to the time of breastfeeding [47].

Lactating mammary glands are an integral part of the mucosal immune system, and the antibodies and cells present in the milk reflect the antigenic stimulation of the Mucosa-Associated Lymphoid Tissue (MALT) in both the intestine and the respiratory tract. The literature reports that antibodies and human milk cells have specificity for a variety of antigens from intestinal and respiratory pathogens [48].

Colostrum differs from most of the secretions by containing viable leukocytes (10^9 cells / ml) during the first days of lactation [49], with amount and activity comparable to blood leukocytes [5]. On the other hand, other defense components present in the secretion that may be associated with protective activity, such as lactoferrin, analogous complexes (receptors), fatty acids (lipids), mucins [50], cytokines and chemokines [51,52,53,54], antibodies such as IgA [55,56], lysozymes [52], probiotics [57], antioxidant factors [58], among other components produced by the maternal immune system, as well as several hormones such as to melatonin [59, 60, 15].

The concentrations of melatonin in human colostrum and mature milk are similar the concentration of this hormone in the bloodstream. Also, immunocompetent colostrum cells can start to produce melatonin after stimuli from injuries, such as those caused by bacteria or fungal metabolites. Studies have shown that lymphocytes and peritoneal macrophages from rats and human colostrum phagocytes produce melatonin in response to activation, and this production of melatonin in the site activates lymphocytes and macrophages to produce IL-12, IL-6, IFN-γ which increases the production of T lymphocytes, the presentation of antigens and the phagocytic activity of macrophages, thus increasing the local inflammatory aspect [61,62,63] and this synthesis of melatonin occurs by the same enzymatic pathway that occurs in human pinealocytes [64].

Immunocompetent breast cells remain highly permeable after childbirth, which makes this type of cell suitable for signaling pathways when collected in a non-invasive manner, suggesting that these cells play an

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https://dx.doi.org/10.22161/ijaers.5.10.2 *ISSN: 2349-6495(P) | 2456-1908(O)*

important role in the protection of the newborn in pathological conditions these cells will be the defense of the newborn, thus consolidating the importance of breastfeeding [62].

IV. MELATONIN

Melatonin is synthesized by the pineal gland [65]. It plays an important role in circadian rhythm control, reproduction, sleep-wake, is directly linked to the regulation of several neuroendocrine axes, protection against cancer and action against free radicals, acting on cell protection [66, 67]. Studies have shown that melatonin may increase the action of innate and acquired immunity and stimulate mainly leukocytes, an immunomodulatory property, which represents an important mechanism of protection for several diseases [9, 68, 8], as well as showing remarkable functional versatility oncotic properties, antioxidants and antiaging [69].

The action of direct melatonin against free radicals has been increasingly studied and its indirect role as an antioxidant has been tested and the effect has been highly effective in reducing oxidative stress in the body when compared to the antioxidants better known as vitamins C and E. Melatonin and its metabolites have positive aspects that make them effective in fighting free radicals. They easily cross the blood-brain and placental barrier, in addition to all maternal organs which leads to greater protection of the placenta and the fetus. Another positive aspect of melatonin is that it can be produced in other compartments, external to the pineal gland [66], and it has been speculated that all cells can synthesize melatonin, mainly in their mitochondria and this local production has the function of protection against radicals free [70].

The production of melatonin by other kinds of cells and organs has been reported, such as the retina, thymus, brain, intestine, bone marrow, ovary, testis, placenta, skin and lymphocytes [71]. High concentrations of melatonin have been found in skin keratinocytes, suggesting that the production of melatonin outside the pineal gland is not only related to the light / dark circadian rhythm, but rather as an antioxidant and anti-inflammatory agent and as a mechanism of stress protection oxidative. This production of melatonin in response to oxidative stress occurs in all living beings, such as plants, unicellular beings, animals and man, and must have been the main function of melatonin in the primitive beings, since they did not have resources in the fight against free radicals.

The production of melatonin by cells of the immune system occurs by activation of pro-inflammatory agents such as cytokines, increases the phagocytic capacity of macrophages and lymphocytes and induces the synthesis of interleukin-2 (IL-2), which has autocrine action and paracrine [69].

Melatonin acts on inflammatory processes and allergic diseases by attenuating the activation of NF-κB, reducing the production of TNF-α and IL-6 and promoting the survival of mast cells via a series of enzyme kinase activation and inhibition processes [72].

Melatonin exerts antioxidant action, which decreases the formation of free radicals, reducing the number of lesions that may affect cellular DNA [73]. It exerts an antiproliferative effect on physiological dose dependent breast cancer MCF-7 (human breast adenocarcinoma cell line) cells, in addition to reducing the rates of invasive and metastatic properties of this cell type [74]. Studies have shown that melatonin decreased cell proliferation and increased expression of p53 and p21 proteins in MCF-7 cells, inhibiting proliferation and inducing apoptosis. The p53 protein is an important tumor suppressor gene and is involved in the regulation of the cell cycle [75]. Melatonin, via activation of the melatonin 1 receptor (MT1) [76], is associated with suppression of growth and development of breast cancer through regulation of growth factors, regulation of gene expression, inhibition of tumor cell invasion and metastasis and by regulation of mammary gland development [77].

V. POLYETHYLENE GLYCOL (PEG) AND THERAPEUTIC APPLICATION IN BIOENGINEERING

Studies aimed at reducing adverse drug effects have been developed as novel therapeutic systems, known as modified release systems [78, 79, 80, 81, 82, 83]. The improvement of the therapeutic properties of bioactive compounds through their incorporation into microcarriers is an important strategy in obtaining new therapies, since cyclical changes in concentration are eliminated; there is biological availability of the compound as well as the reduction in toxicity, number of administered doses and suppression of adverse reactions [84].

PEG-drug conjugates and microemulsions-drug, are being studied as possible modified release systems for a variety of molecules and drugs [85, 86, 87, 88, 89, 90, 91, 92, 83]. This combination has many advantages such as prolonged residence in the organism, decreased degradation by metabolic enzymes and reduction or elimination of the immunogenicity of proteins [87].

Several studies have shown that the association of PEG microspheres with molecules, hormones or proteins increases the immunomodulatory capacity of both blood and colostrum phagocytes and suggests that the adsorption of these compounds to PEG microspheres has

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https://dx.doi.org/10.22161/ijaers.5.10.2 *ISSN: 2349-6495(P) | 2456-1908(O)*

immunostimulatory effects and can be considered a important material/vehicle, with potential for future therapeutic applications in infectious diseases or tumors [13, 15, 14, 17, 16, 93].

Studies using hormones such as melatonin and secretory IgA antibodies extracted from human milk adsorbed onto PEG microspheres showed that the controlled release of this compound was able to reduce viability and induce apoptosis in MCF-7 cell lines [11,12]. Other herbicidal and barium chloride studies, adsorbed to PEG microspheres on human blood mononuclear cells co-cultured with breast cancer cell lines (MCF-7), showed a pro-apoptotic effect in breast cancer cells MCF-7 human [94, 95]. Immunotherapy for tumor treatments based on cytotoxic properties of immunocompetent cells has also been the focus of many studies. Both T cells and phagocytic cells are considered effectors with antitumor activity [96]. The melatonin adsorbed to the PEG microsphere was able to increase the functional activity of colostrum phagocytes and that this modified hormone release system may represent an alternative in the treatment of diseases [17].

Here we hypothesize that melatonin adsorbed on PEG microsphere may be effective in the treatment of breast cancer. The possible interactions between components present in human milk and therapy of bioengineered melatonin as a strategy for the prevention and treatment of breast cancer are shown in figure 1.

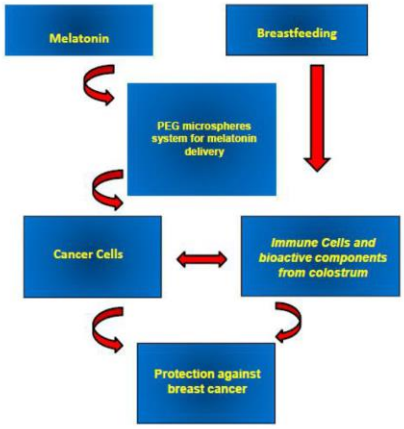

Fig.1: Hypothetical model of therapy with Melatonin bioengineered

Additionally the chronotherapy, the practice of drug administration according to the circadian rhythm, is intended to maximize efficacy and minimize toxicity in the body. This approach has proven advantageous in several diseases, such as cancer and asthma [97] and has been an alternative of treatments based on melatonin.

On the other hand, the expected response by chemotherapy, radiotherapy, hormone treatments, is the induction of apoptosis, since the balance between cell proliferation and apoptosis is determinant for its growth [98]. After initiation of chemotherapy, within 24 h there is a significant increase in apoptosis in breast tumors, associated with decreased proliferation. This response may occur differently in the various tumor types, and at the end of the chemotherapies there is an increase in the levels of the anti-apoptotic Bcl-2 protein, which favors the occurrence of chemoresistant residual cells, which may be important for tumor recurrences [99].].

The anti-apoptotic role in normal and pro-apoptotic cells in cancer cells has been reported in studies with melatonin, giving prominence to this hormone, since conventional cancer treatments can not do this discrimination between healthy cells and cancer cells, placing it in a prominent position in the search for effective treatment against various types of cancer, and especially against breast cancer [100].

VI. CONCLUSION

Major advances in cancer therapy have been occurring, and the study of the use of melatonin in cell culture or in vivo oncology has shown promise. The mechanisms of action of melatonin in reducing oxidative stress and the activation of apoptosis in cancer cells has put this hormone as a highlight in the adjuvant use of cancer treatment.

And considering that the breast tissue is in constant and direct contact with the soluble and cellular immune components in the milk, and the numerous immunological constituents of the breast milk, among these high concentrations of melatonin, macrophages, it is possible that interactions of these components, directly or modified release systems with factors present in tumor cells may be an alternative for tumor immunotherapy.

There is still much to study and develop to further increase the cure rates of cancer patients, as well as eradicate the occurrence of adverse reactions that both discomfort and incapacitate the patient, often leading to withdrawal of treatment. There is a need to improve the studies around melatonin as an immunomodulatory agent of colostrum phagocytes in the action against breast cancer cells, since these cells are present in large quantities, mainly in women who have breastfed, which can increase even more the chances of prevention against breast cancer.

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5.2 Artigo 2: Submetido na revista Chronobiology International.

Title: Time-dependent immune effects of melatonin on colostrum cells co-cultured with human breast cancer cells

Running title: Melatonin effects in colostrum and cancer cells

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ABSTRACT

The aim of this study was to investigate the effect of melatonin adsorbed to PEG microspheres during different phases of the day on oxidative stress and apoptosis in human colostrum mononuclear (MN) cells co-cultured with breast cancer tumor cells (MCF-7). The MCF-7 cells were obtained from the American Type Culture Collection, and the MN cells were obtained from volunteer donors. The cells were preincubated for 24 h with or without 100 $ng.mL^{-1}$ melatonin (MLT), PEG microspheres (PEG) or 100 $ng.mL^{-1}$ MLT adsorbed to PEG microspheres (PEG-MLT). Superoxide release, superoxide dismutase (SOD), intracellular calcium release and apoptosis in the MN cells, MCF-7 cells and co-cultures were determined. The MN cells and co-cultured cells during the nocturnal period and the MCF-7 cells during the diurnal period exhibited increased superoxide release in the presence of PEG-MLT. MN cells treated with MLT during both periods of the day exhibited the highest SOD concentrations, whereas the MCF-7 cells had high SOD levels when incubated with PEG-MLT during the nocturnal period. The nocturnal period co-culture, independent of treatment, showed the highest levels of the enzyme. The highest amount of intracellular Ca^{2+} release was observed in MN cells and MCF-7 cells co-cultured with PEG-MLT during the nocturnal period. Irrespective of the phase of day, the highest apoptosis index was observed in co-cultures of cells incubated with MLT-PEG. These data suggest that melatonin-modified release plus physiological melatonin produced in higher concentrations during the night can increase the effective activity of this hormone against tumors.

Key words: Breast cancer, Immune resposnse; Melatonin; colostrum MN cells; MCF-7.

INTRODUCTION

Clinical and experimental evidence has supported the hypothesis that colostrum is important in the protection against breast cancer (1, 2) and can reduce the risk of disease in women who breastfeed (3, 4). Colostrum contains soluble and cellular components, such as lipids, carbohydrates, proteins, viable leukocytes (particularly macrophages) (5), and hormones, which are important for immune defense (6, 7) and exhibit a circadian rhythm (8).

Melatonin, one of the hormones present in milk that is produced by the pineal gland (6, 9), in colostrum mononuclear cells (10), exhibits a pronounced circadian rhythm, has an important role in the control of the sleep-wake cycle, and is directly connected to the regulation of several neuroendocrine axes (11).

Many of the benefits of melatonin and its metabolites are related to their antioxidant, anti-inflammatory (12, 13) and pro-oxidative effects (14). Oxidative stress, chronic inflammation and cancer are closely linked. Studies have shown the action of melatonin against free radicals (11, 15). It is known that oxidative stress can lead to chronic inflammation, which in turn can mediate most chronic diseases, among them cancer (16).

Melatonin has anti-tumor effects on human MCF-7 breast cancer cells, being able to increase apoptosis in these cells, likely because of modifications to intracellular Ca^{2+} release (17). However, it known that the oral bioavailability of melatonin is less than 20% due to extensive first pass hepatic metabolism and variable rates of absorption (18-20). Thus, PEG-drug conjugates are being used as possible modified release systems for a variety of molecules and drugs (21-28).

The administration of drugs adsorbed to PEG microspheres has been an alternative treatment for a number of diseases (29), including breast cancer. PEG microspheres are a promising agent for the delivery of the hormone melatonin, as they can prevent its degradation and increase its bioavailability within an organism, decrease degradation via metabolic enzymes and reduce or eliminate the immunogenicity of proteins (23). The PEG microspheres when combined with melatonin, has shown to reduce cell viability and to induce apoptosis in breast cancer tumor cell lines (17).

Additionally, regarding disease infections, studies have reported different effects of melatonin depending on the phase of day (7,15) and on the actions associated with PEG microspheres in human colostrum phagocytes (30); despite the influence of this hormone on anti-tumor mechanisms, the effects of interactions of colostrum MN cells and melatonin during different phases of the day on tumor cells have not yet been elucidated.

The action of melatonin on human cells suggests that this hormone exerts effects differently depending on time. Determining the best time for the actions of this hormone is important in order to allow adequate treatment, and this information can be the basis of possible therapeutic techniques. Additionally, the immunomodulatory potential of melatonin on colostrum cells in the initiation of tumor cell killing should be considered in order to clarify its possible use as a treatment for breast cancer. The aim of this study was to investigate the effect of melatonin adsorbed to PEG microspheres on oxidative stress and apoptosis in human colostrum MN cells co-cultured with breast cancer tumor cell lines (MCF-7).

MATERIALS AND METHODS

Subjects

This cross-sectional study evaluated 60 mothers clinically healthy who had no diagnosed diseases, such as hypertension and diabetes, and who reported not consuming alcoholic beverages or tobacco at the Municipal Hospital of Barra do Garças, Mato Grosso state, Brazil. The volunteers signed an informed consent form before entering the study, which was approved by the local ethics committee Araguaia (Protocol Number CAAE: 45102815.3.0000.5587). The reported experiments human are in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration.

Polyethylene glycol (PEG) microsphere preparation

The microspheres were obtained from polyethylene glycol (PEG) 6000 using a modification (28) of a previously described protocol (31). Briefly, 20 g of PEG 6000 was resuspended in 100 mL of a 2% sodium sulfate solution in phosphate-buffered saline (PBS) and incubated at 37°C for 45 min. After incubation, the PEG microspheres were diluted 3:1 in PBS and washed twice in PBS (500 x g, 5 min). The PEG microspheres were resuspended in PBS. The formation of the microspheres was thermally induced by subjecting the solution to 95°C for 5 min. For adsorption, the suspensions of PEG microspheres in PBS were incubated with MLT (Sigma, St. Louis, USA; concentration 100 ng/mL) at 37°C for 30 minutes. After this period the PEG microspheres with adsorbed MLT was washed twice in PBS (500 x g, 5 min). The loading efficient of adsorption the PEG microspheres with adsorbed melatonin (MLT) were then analyzed by fluorescence microscopy using a fluorescently labeled with a solution of Dylight-488 (Pierce Biotechnology, Rockford, USA; 10 µg/mL) (30).

Obtaining colostrum samples and colostrum cells separation

Colostrum from each woman was collected in sterile plastic tubes. The collection of colostrum from the lactating participants was performed following the

protocol developed by França et al. (8). Two breast milk samples were collected (diurnal and nocturnal) from each mother at 3 days postpartum (colostrum) for a total of 120 samples.

The samples were centrifuged (160 x g, 4°C) for 10 min. Cells were separated by a Ficoll-Paque gradient (Pharmacia, Upsala, Sweden). The cells were adjusted at a final concentration of $2x10^6$ cells/ml.

Colostrum MN cells treatment

Colostrum MN cells (diurnal and nocturnal collection) were pre-incubated for 24 h with or without 50 µL of melatonin (MLT -100 ng/mL final concentration) (7), 50 µL of polyethylene glycol (PEG) microspheres or 50 µL of melatonin adsorbed in PEG microspheres (PEG-MLT - 100 ng/mL final concentration). Next, the cells were diluted in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA), penicillin (20 U/mL) and streptomycin (20 µg/mL) (Sigma, St. Louis, MO, USA) at 37°C for 24 h in a humid atmosphere containing 5% CO_2.

MCF-7 cell culture

MCF-7 human breast cancer cells were obtained from American Type Culture Collection (ATCC, USA). The cells were maintained as monolayer cultures in 75 cm^2 plastic culture flasks in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA), penicillin (20 U/mL) and streptomycin (20 µg/mL) (Sigma, St. Louis, MO, USA) at 37°C in a humid atmosphere containing 5% CO_2. The cells were subcultured every 5±2 days.

MCF-7 Cell treatment

Subconfluent (80%) monolayers of MCF-7 cells were treated with a concentration of trypsin (Sigma, St. Louis, USA) adjusted for $5x10^6$ cells/mL. Then, the trypsinized cells were pre-incubated for 24 h with or without 50 µL of MLT (100 ng/mL

final concentration), 50 µL of PEG microspheres (PEG) or 50 µL of MLT adsorbed in PEG microspheres (PEG-MLT) (100 ng/mL final concentration). Next, the cells were diluted in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA), penicillin (20 U/mL) and streptomycin (20 µg/mL) (Sigma, St. Louis, MO, USA) at 37°C for 24 h in a humid atmosphere containing 5% CO_2. The cells were cultured in two periods (diurnal and nocturnal) according to colostrum collection.

Co-culture of MN cells and MCF-7 cells

Cell co-culture systems incorporating MN and MCF-7 cells ($5x10^6$ cells/mL) (32) were performized using the same protocol describe above.

Release of superoxide anion

Superoxide release was determined by cytochrome C (Sigma, ST Louis, USA) reduction (7, 33). Briefly, MN cells, MCF-7 cells and co-culture were treated according to the describe above. The suspensions were then resuspended in PBS containing 2.6 mM $CaCl_2$, 2 mM $MgCl_2$, and cytochrome C (Sigma, ST Louis, USA; 2 mg/mL). The suspensions (100 µL) were incubated for 60 min at 37°C on culture plates. The reaction rates were measured by absorbance at 550 nm, and the results were expressed as nmol/O^{2-}. All experiments were performed in duplicate.

CuZn-superoxide dismutase determination (CuZn-SOD – E.C.1.15.1.1)

Analysis of the CuZn-SOD enzyme in culture supernatant of MN, MCF-7 and co-culture was performed using the nitroblue tetrazolium (NBT) reduction method (Sigma). The individual samples were placed in glass tubes, with another tube containing a standard solution. Each tube contained 0.5 mL of the sample, and the standard tube contained 0.5 mL of hydro-alcoholic solution. Next, 0.5 mL of chloroform-ethanol solution (1:1 ratio) and 0.5 mL of reactive mixture (NBT increased by EDTA) was

added to the tubes. The experimental and standard solutions received 2.0 mL of buffer carbonate, and the pH was increased to 10.2 after the addition of hydroxylamine. The tubes remained still at room temperature for 15 min and were subsequently read at 560 nm (34**).**

Intracellular Ca^{2+} release determination

Cells were loaded with the fluorescent radiometric calcium indicator Fluo3-acetoxymethyl (Fluo3-AM; Sigma, St. Louis, USA). Cell suspensions were incubated with 5 µL of Fluo-3 (1µg/mL) for 30 min at 37°C. After incubation, the cells (MN cells or/and MCF-7 cells) were washed twice in PBS containing BSA (5mg/mL; 160 x g, 10 min, 4°C) and then analyzed by flow cytometry (FACSCalibur system; BD, San Jose, USA). Fluo-3 was detected using a 530/30 nm filter for intracellular Ca^{2+}. The rate of intracellular Ca^{2+} release was expressed as the geometric mean fluorescence intensity of Fluo-3.

Apoptosis assay

Untreated cells were used as negative controls, and cells treated with staurosporine (Sigma, St. Louis, USA) were used as positive controls. The cells (MN cells or/and MCF-7 cells) were resuspended in 500 µL of binding buffer containing 5 µL of annexin V-FITC (Annexin V-FITC Apoptosis Detection Kit, Alexis™, San Diego, USA) and 5 µL of PI and then incubated for 10 min at room temperature. The fluorescence of the cells was analyzed by flow cytometry (FACSCalibur system; BD, San Jose, USA). The obtained data were analyzed using CellQuest software. The cells were classified as follows: viable cells ($annexin^-/PI^-$), early apoptotic cells ($annexin^+/PI^-$), late apoptotic cells ($annexin^+/PI^+$), and necrotic cells ($annexin^-/PI^+$).

Statistical analysis

An analysis of variance (ANOVA) was used to evaluate superoxide release, culture supernatant SOD levels, intracellular calcium and apoptosis index of cells (MN, MCF-7 and MN/MCF-7) in the presence or absence of PEG microspheres with adsorbed melatonin. Statistical significance was considered when $P<0.05$.

RESULTS

The effect of melatonin adsorbed to PEG microspheres on superoxide release

Melatonin increased the release of superoxide from colostrum phagocytes compared to the spontaneous release. Additionally, independent of the phases of day, the phagocytes exposed to melatonin adsorbed to PEG microspheres displayed increased superoxide release when compared to the phagocytes exposed to the PEG microspheres alone. Furthermore, the release of superoxide during the diurnal period decreased significantly in the presence of PEG-MLT (Figure 1A).

The MCF-7 cells showed higher superoxide release when treated with melatonin during both phases of day when compared to untreated cells. Superoxide release during the nocturnal period decreased significantly in the presence of melatonin adsorbed or not to PEG microspheres (Figure 1B).

Melatonin increased the release of superoxide by cells in co-culture. The MN cells in co-culture with the MCF-7 cells exposed to melatonin adsorbed to PEG microspheres during the nocturnal period displayed increased superoxide release (Figure 1C).

The effect of melatonin adsorbed to PEG microspheres on the superoxide dismutase (SOD) enzyme

MN cells during both periods of the day exhibited highest SOD concentrations in culture supernatant when the cells were treated with MLT. The PEG-MLT increased the SOD concentration in the culture supernatant of MN cells during the nocturnal period and decreased the concentration during the diurnal period (Table 1).

MCF-7 cells incubated with MLT and PEG-MLT during the nocturnal period had high SOD concentration, whereas during the diurnal period, the cells treated with PEG-MLT exhibited a decreased concentration. The enzyme concentration in the culture supernatant of the MCF-7 treated with MLT or MLT adsorbed to PEG microspheres was significantly higher during the nocturnal period than during the diurnal period (Table 1).

During the diurnal period, MN cells and MCF-7 cells in co-culture showed lower SOD levels when they were incubated with PEG microspheres. Higher SOD concentrations were observed when the co-culture was treated with MLT adsorbed or not to the PEG microspheres during the nocturnal period (Table 1). A comparison of the periods showed that the MN cells in co-culture with the MCF-7 cells, independent of treatment, showed the highest levels of SOD concentration during the nocturnal period compared to the diurnal period (Table 1).

The effect of melatonin adsorbed to PEG microspheres on intracellular Ca^{2+} release

Table 2 shows the rate of intracellular Ca^{2+} release in MN cells, MCF-7 cells and co-culture of MN and MCF-7 cells. The PEG microspheres reduced the release of intracellular Ca^{2+} from the MN cells during the diurnal period. When these cells were incubated with MLT, independent of the phases of day, they showed higher intracellular Ca^{2+} release than the non-treated cells did. The highest intracellular Ca^{2+} release was observed in MN cells treated with MLT during the diurnal period. The MLT adsorbed to PEG microspheres increased intracellular Ca^{2+} release in the MN cells during the nocturnal period (Table 2).

MCF-7 cells exhibited low spontaneous intracellular Ca^{2+} release. When these cells were incubated with MLT or MLT adsorbed to PEG microspheres during the diurnal period, they exhibited increased intracellular Ca^{2+} levels (Table 2).

In co-culture, independent of the day period, the cells showed higher intracellular Ca^{2+} release when they were incubated with MLT adsorbed or not to PEG microspheres. The highest intracellular Ca^{2+} was observed during the nocturnal period when the cells were treated with MLT adsorbed or not to PEG microspheres (Table 2).

The effect of melatonin adsorbed to PEG microspheres on apoptosis rates

To evaluate apoptosis induction in MN cells, MCF-7 cells and co-culture (MN and MCF-7 cells) cells were stained with annexin V and analyzed using flow cytometry (Table 3, Figure 2 and Figure 3).

The apoptosis index of the MN cells was similar when these cells were incubated with PEG but increased in terms of necrosis during the diurnal period. MLT and MLT-PEG, independent of the period of day, increased the apoptosis rates (Table 3, Figure 2 and Figure 3). A comparison of the periods showed that nocturnal MN phagocytes treated with MLT or PEG-MLT exhibited decreased necrosis in the MN cells (Table 3).

The apoptosis rate was lower in the non-treated MCF-7 cells than in the treated MCF-7 cells. The PEG microspheres increased apoptosis in MCF-7 cells during the diurnal period and necrosis during both periods. The apoptosis rates, independent of the phase of day, increased in the cells that were treated with MLT, and necrosis increased during the diurnal period. MCF-7 treated with PEG-MLT exhibited increased apoptosis during both periods and increased necrosis during the diurnal period (Table 3). A comparison of the periods in regard to the non-treated MCF-7 showed higher apoptosis rates during the nocturnal period than during the diurnal period (Table 3, Figure 2 and Figure 3). When these cells were treated with MLT adsorbed or not, they showed decreased necrosis during the nocturnal period (Table 3).

In general, the apoptosis rate in the co-cultures of MN cells and MCF-7 cells was increased (Figure 2 and Figure 3). During the diurnal period, the co-culture of cells with

MLT and PEG-MLT increased the apoptosis rates during both of the periods that were evaluated. Irrespective of the phase of day, the highest apoptosis index was observed in the co-cultures of cells incubated with MLT-PEG microspheres. The PEG-MLT decreased the necrosis index in the co-culture of cells during the nocturnal period (Table 3). Co-culture in the presence of MLT exhibited lower necrosis during the nocturnal period than during the diurnal period (Table 3).

DISCUSSION

Breast milk itself undergoes chronobiological changes in cell immunity. The composition of breast milk represents very important environmental influences on human health. These factors can change the progression of diseases, as well as their longevity (35). The immune system is influenced by nutrition and some nutrients can be directly regulated by signaling pathways, while some dietary factors have a regulatory influence on hormones that, in turn, modulate the immune system (36**)**. In this study, melatonin adsorbed to PEG microspheres exhibited anti-tumor activity against MCF-7 human breast cancer cells, especially when in co-culture with colostrum MN cells, and this activity oscillated as a function of the time of day.

Progress has been made in the understanding of circadian variations and of the cell functions (7,37). Chronotherapy is an underused practice of delivering therapy at optimal times in order to maximize efficacy and minimize toxicity; however, this approach has been shown to be advantageous in various diseases, such as cancer (38). Polymeric matrices associated with hormones have increased the possibility of obtaining new drugs to activate cells and act on tumor cells. The generation of free radicals is an important mechanism of protection for the organism (39,40).

In this work, we showed that both the release of superoxide from colostrum MN phagocytes and the SOD concentration increased during both phases of day in

association with melatonin. The adsorption of melatonin into PEG increased superoxide and SOD, and this effect was more evident during the nocturnal period than during the diurnal period, suggesting a balance in the mechanisms of cellular oxidative stress. It was interesting that the MCF-7 cells treated with MLT or PEG-MLT exhibited an increase in superoxide release during the diurnal period, while higher concentrations of SOD were observed during the nocturnal period. Most likely, this imbalance in cellular oxidative metabolism may be associated with tumor cell survival. Similar results with other cell lines also suggested the involvement of an oxidative imbalance in target cells (41).

In co-culture, the cells treated with PEG-MLT exhibited an increase in superoxide release and the highest levels of SOD concentration during the nocturnal period, suggesting that the antioxidant system presents variations depending on the phase of day and that variations in the oxidative mechanisms of colostrum MN cells remain, and most likely, the maintenance of this oxidative balance may be auxiliary in the control of tumor cells.

Some studies have reported that the effects of melatonin administration are different depending on whether the pineal gland is present (42,43) and have suggested that the presence of a daily rhythm in terms of melatonin may affect the sensitivity of specific target tissue to this hormone and may be most effective during the nocturnal period because tissues are most sensitive to the hormone at this time (43). In this study, we confirmed that the effects of melatonin adsorbed to PEG microsphere on oxidative metabolism in MN cells were more evidence during the nocturnal period.

Previous studies showed that, in MCF-7 cell cultures, periodic exposure to melatonin was as effective as continuous exposure in terms of antiproliferative effects, although the amount of melatonin and the time of cell exposure to the hormone were obviously half when under pulsatile exposure (43). Here, considering the phases of day,

we observed that, in co-culture rather than in isolated cells, the treatment of melatonin adsorbed to PEG microspheres was capable of acting on the oxidative cellular mechanisms.

Some studies of free radical regulatory mechanisms have suggested that superoxide dismutase plays a protective role (44). However, the fact that melatonin follows a day-night variation makes it an important immunomodulatory agent that can improve cell activation processes. Melatonin has an influence on the activity and the expression of superoxide dismutase, under both physiological and elevated oxidative stress (39), and acts on scavenged free radicals (45), suggesting that the actions of melatonin can be considered in support of its possible antitumoral effects.

Free radical generation has been reported to be an important mechanism for protecting the body in various processes (46,47) and physiological signaling pathways (48). Excessively high levels of free radicals cause damage to cellular proteins, membrane lipids and nucleic acids (49) and increase DNA damage and ATP depletion, leading to the induction of apoptosis (50,51).

Cancer development and progression is characterized by dynamic changes in the expression and function of protein kinases (52) or their signaling pathways, which can lead to malignant transformation of breast cells (53). Studies have reported that a modified release system with antibodies (54) or melatonin (17) are capable of activating the signaling pathways involving calcium intracellular mechanisms and of inducing apoptosis in breast cancer cells.

In this study, the intracellular calcium showed chronobiological variation in the different cells evaluated. In MN cells, melatonin increased the intracellular calcium during both periods. In the modified release system, this hormone was capable of increasing the calcium during the nocturnal period. This variation was different in the

MCF-7 cells since high levels of intracellular calcium were observed during the diurnal period, suggesting modified actions of melatonin on the MCF-7 cells. Melatonin introduced to the co-culture increased intracellular calcium release with a profile similar to that of the MN cells, which can be interpreted as immunostimulatory action towards immune cells in co-culture with malignant cells.

Alterations in the intracellular Ca^{2+} influx in human cells may cause cellular damage that eventually culminates in the activation of cell death pathways (55), and the excessive release of intracellular Ca^{2+} has been associated with apoptosis induction in human cells (17, 54, 56). In this work, independent of the phases of day, the highest levels of apoptosis were observed in cells in the presence of melatonin adsorbed to the PEG microsphere, and the most expressive apoptosis was observed in the co-cultures. The MN cells, despite the increase in apoptosis in the presence of the treatment, maintained viability indices regardless of the time of day, specifically above 80%. Interestingly, during the nocturnal period, a lower index of necrosis was observed, which may be associated with the effects of endogenous melatonin. Further studies are needed to confirm the involvement of endogenous melatonin in the process of necrosis.

The role of melatonin as an antiproliferative agent in breast cancer has been extensively studied, showing that melatonin reduces the invasive and metastatic properties of MCF-7 cells (57,58). Studies have shown that melatonin decreased cell proliferation and increased expression of p53 and p21 proteins in MCF-7 cells (59) which are proteins that are capable of inducing apoptosis (59).

The importance of the period of administration and of the use of the modified system of melatonin for the development of future anti-tumor therapies should be considered since the PEG microsphere system increases the bioavailability of hormones in terms of both concentration and time (17).

In the present study, the PEG microspheres were shown to be an important melatonin vehicle that were capable of improving immune responses through the activation of colostrum cells to act on breast tumor cells. This polymer has important properties that modulate and prolong the action of melatonin when this drug is administered orally, and it seems to be more effective during the nocturnal period, particularly under the activation of antioxidant systems.

In conclusion, these data suggested that a melatonin-modified release and, most likely, physiological melatonin produced in higher concentrations during the nocturnal period can potentialize the activity of this hormone in being more effective against tumors. In addition, an increase in apoptosis and the imbalance in oxidative status in the cancer cells were observed, as a result of the immunomodulatory effect of melatonin in co-cultures of colostrum cells and human breast cancer cells. These results implied that chronobiological variations in soluble and cellular components present in the colostrum can act as anti-tumor agents during different phases of the day. However, clinical tests during the nocturnal period must be performed before application of melatonin as a supplementary agent for therapeutic use in order to confirm the efficacy of this treatment.

ACKNOWLEDGMENTS

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq Nº: 447218/2014-0; Nº: 308600/2015-0), Brazil.

DECLARATION OF INTEREST STATEMENT

The authors declare no conflict of interest and non-financial competing interests regarding the publication of this article.

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Table 1 CuZn-superoxide dismutase concentrations in the culture supernatants from MN cells collected during both the diurnal and nocturnal periods, from MCF-7 incubated during the diurnal and nocturnal periods, and from co-cultures (MN cells and MCF=7 cells during both periods).

SOD (IU)	Phases	RPMI Medium	PEG	MLT	PEG-MLT
MN cells	Diurnal	37.5 ± 5.7	24.5 ± 7.9*	41.3 ± 4.4*	21.0 ± 6.0*$^{\#}$
	Nocturnal	19.5 ± 2.1†	19.9 ± 2.7	31.7 ± 3.8*†	26.3 ± 3.8*$^{\#}$
MCF-7 cells	Diurnal	35.7±1.8	13.9±3.8*	34.1±3.5	24.5±6.3*$^{\#}$
	Nocturnal	37.6±4.3	38.8±2.2†	45.9±1.5*†	49.0±2.5*†
Co-culture	Diurnal	14.0±4.1	5.1 ± 1.9*	9.6 ± 1.7	9.8 ± 2.4
	Nocturnal	18.4±5.3	24.2 ± 2.4†	28.1 ± 2.4*†	30.2 ± 2.5*†

International units (IU); RPMI1640 medium; polyethylene glycol (PEG) microsphere; melatonin (MLT); melatonin adsorbed to the PEG microspheres (PEG-MLT). The results represent the mean ± SD of 10 experiments. *$p<0.05$: comparing non-treated cells to cells treated with MLT or PEG-MLT, considering the same collection period (diurnal or nocturnal); $^{\#}p<0.05$: comparing MLT and PEG-MLT, considering the same collection period (diurnal or nocturnal); $^{\dagger}p<0.05$ comparing superoxide release between the different collection periods (diurnal or nocturnal), considering the same treatment.

Table 2 Release of intracellular Ca^{2+} from the MN cells, MCF-7 cells and co-cultures (MN cells Intracellular Ca^{2+} release is represented by mean fluorescence intensity as determined by flow cytometry.

Intracellular Ca^{2+}	Phases	PBS	PEG	MLT	PEG-MLT
MN cells	Diurnal	12.5 ± 5.6	5.7 ± 2.2*	19.5 ± 1.0*	15.6 ± 1.5
	Nocturnal	4.8 ± 2.5†	5.4 ± 2.6	10.9 ± 3.7*†	16.3 ± 3.1*
MCF-7 cells	Diurnal	5.6 ±1.7	6.7±1.5	21.5±3.4*	27.1±8.6*
	Nocturnal	5.4±0.4	6.1±0.4	6.0 ±0.8†	16.1±3.1*$^{\dagger\#}$
Co-culture	Diurnal	5.3±1.3	18.6 ± 1.9*	21.7 ± 4.7*	16.4 ± 4,5*
	Nocturnal	6.7±1.3	6.1 ± 1.6†	16.1 ± 1.4*†	25.9 ± 1.4*†

The results represent the mean ± SD of five experiments. Polyethylene glycol (PEG) microspheres; melatonin (MLT); melatonin adsorbed to PEG microspheres (PEG-MLT). Q1: Viable cells ($annexin^-/PI^-$); Q2 ($annexin^+/PI^-$) and Q3 ($annexin^+/PI^+$): total apoptotic cells; Q4: necrotic cells ($annexin^-/PI^+$).*$p<0.05$: treated cells compared with non-treated cells; $^{\#}p<0.05$: comparing the different treatments (MLT and PEG-MLT); $^{\dagger}p<0.05$ comparing different collection period (diurnal or nocturnal), considering the same treatment.

Table 3. Apoptosis (%) and necrosis (%) of colostrum Mononuclear (MN) Cells, MCF-7 Cells and co-culture of colostrum MN Cells and MCF-7 Cells in the presence of MLT adsorbed to PEG microspheres.

	Phases	**Viables**	**Apoptosis**	**Necrosis**
		Q1	**(Q2+Q3)**	**Q4**
MN	Diurnal	92.9 ± 3.6	6.7 ± 3.7	0.4 ± 0.2
	Nocturnal	85.1 ± 5.2	7.3 ± 1.5	0.8 ± 0.1
MN +PEG	Diurnal	80.7 ± 2.5	9.2± 2.9	10.2 ± 1.8*
	Nocturnal	86.7 ± 4.9	6.2± 3.8	0.9 ± 0.4†
MN +MLT	Diurnal	84.2± 2.3	13.9 ± 2.1*	2.0 ± 0.3*
	Nocturnal	81.0 ± 4.7	16.1 ± 4.8*	0.6 ± 0.4†
MN+PEG- MLT	Diurnal	83.1 ± 8.9	18.9 ± 7.7*	3.0 ± 0.9*
	Nocturnal	82.0 ± 3.8	16.5 ± 2.7*	0.3 ± 0.1†
M CF-7	Diurnal	97.3 ± 0.8	0.4 ± 0.1	2.3 ± 0.4
	Nocturnal	63.5 ± 6.7†	15.3 ± 0.7†	2.4 ± 1.2
MCF-7+PEG	Diurnal	80.6 ± 0.9	4.9 ± 1.4*	14.5 ± 0.9*
	Nocturnal	57.9 ± 8.9†	15.8 ± 3.2†	6.2 ± 0.4*†
MCF-7+MLT	Diurnal	58.8 ± 3.1*	36.0 ± 2.8*	5.2 ± 3.2*
	Nocturnal	40.4 ± 8.5†	35.1 ± 9.5*	1.0 ± 0.5†
MCF-7+PEG- MLT	Diurnal	31.7 ± 3.2*#	57.2 ± 6.4*$^{\#}$	6.0 ±1.1*$^{\#}$
	Nocturnal	51.1 ± 5.9$^{\dagger *}$	47.5 ± 4.3*	0.7 ± 0.4*†
MN+ MCF-7	Diurnal	65.7 ±7.9	24.4 ± 7.7	9.9 ± 1.6
	Nocturnal	56.7 ± 10.0	20.9 ± 4.1	4.5 ± 2.0†
MN+ MCF-7+PEG	Diurnal	60.1 ± 2.7	30.7 ± 5.9	9.2 ± 2.8
	Nocturnal	52.9 ± 10.4	18.9 ± 4.0†	8.2 ± 1.6
MN+ MCF-7+MLT	Diurnal	50.6 ±6.7	40.6 ± 6.5*	8.9 ± 2.7
	Nocturnal	56.7 ± 6.2	48.3 ± 1.6*	4.9 ± 0.8†
MN+MCF-7+PEG-MLT	Diurnal	33.6 ± 4.7*$^{\#}$	60.7 ± 3.6*$^{\#}$	5.8 ± 1.5
	Nocturnal	33.0 ± 20.9*$^{\#}$	62.8 ± 20.7*$^{\#}$	2.5 ± 0.4$^{\dagger\#}$

The results represent the mean ± SD of five experiments. Polyethylene glycol (PEG) microspheres; melatonin (MLT); melatonin adsorbed to PEG microspheres (PEG-MLT). Q1: Viable cells (annexin^{-}/PI^{-}); Q2 (annexin^{+}/PI^{-}) and Q3 (annexin^{+}/PI^{+}): total apoptotic cells; Q4: necrotic cells (annexin^{-}/PI^{+}).*$p<0.05$: treated cells compared with non-treated cells; $^{\#}p<0.05$:

comparing the different treatments (MLT and PEG-MLT); †p<0.05 comparing different collection period (diurnal or nocturnal), considering the same treatment.

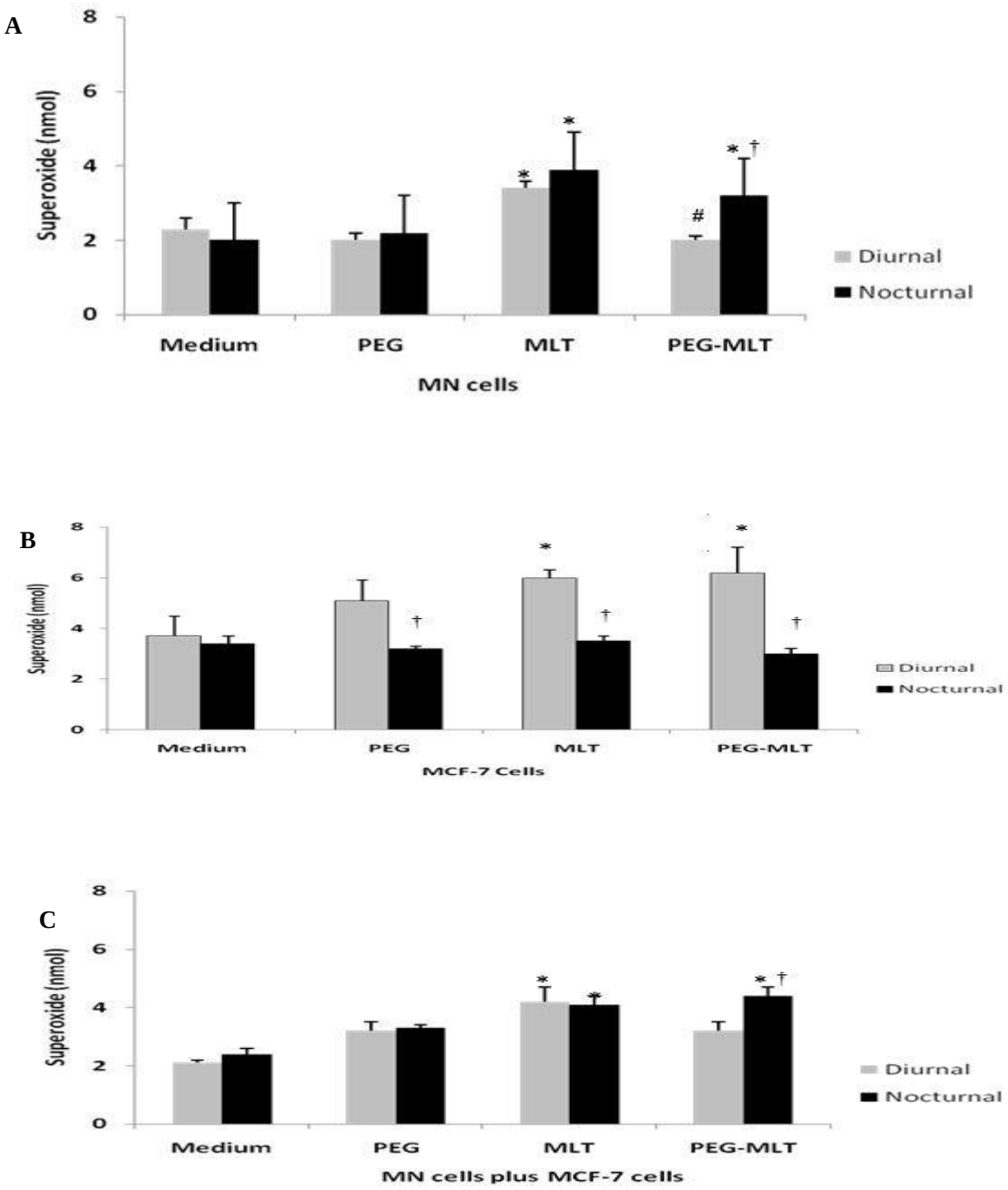

Figure 1. Superoxide release from the colostrum MN cells, MCF-7 cells and co-cultures (colostrum MN and MCF-7 cells). The results represent the mean ± SD of 10 experiments. RPMI1640 medium; polyethylene glycol (PEG) microspheres; melatonin (MLT); melatonin adsorbed to the PEG microsphere (PEG-MLT). *p<0.05: comparing spontaneous release from non-treated cells to cells treated with MLT or PEG-MLT, considering the same collection period (diurnal or nocturnal); #p<0.05: comparing MLT

and PEG-MLT, considering the same collection period (diurnal or nocturnal); †p<0.05 comparing superoxide release between the different collection periods (diurnal or nocturnal), considering the same treatment

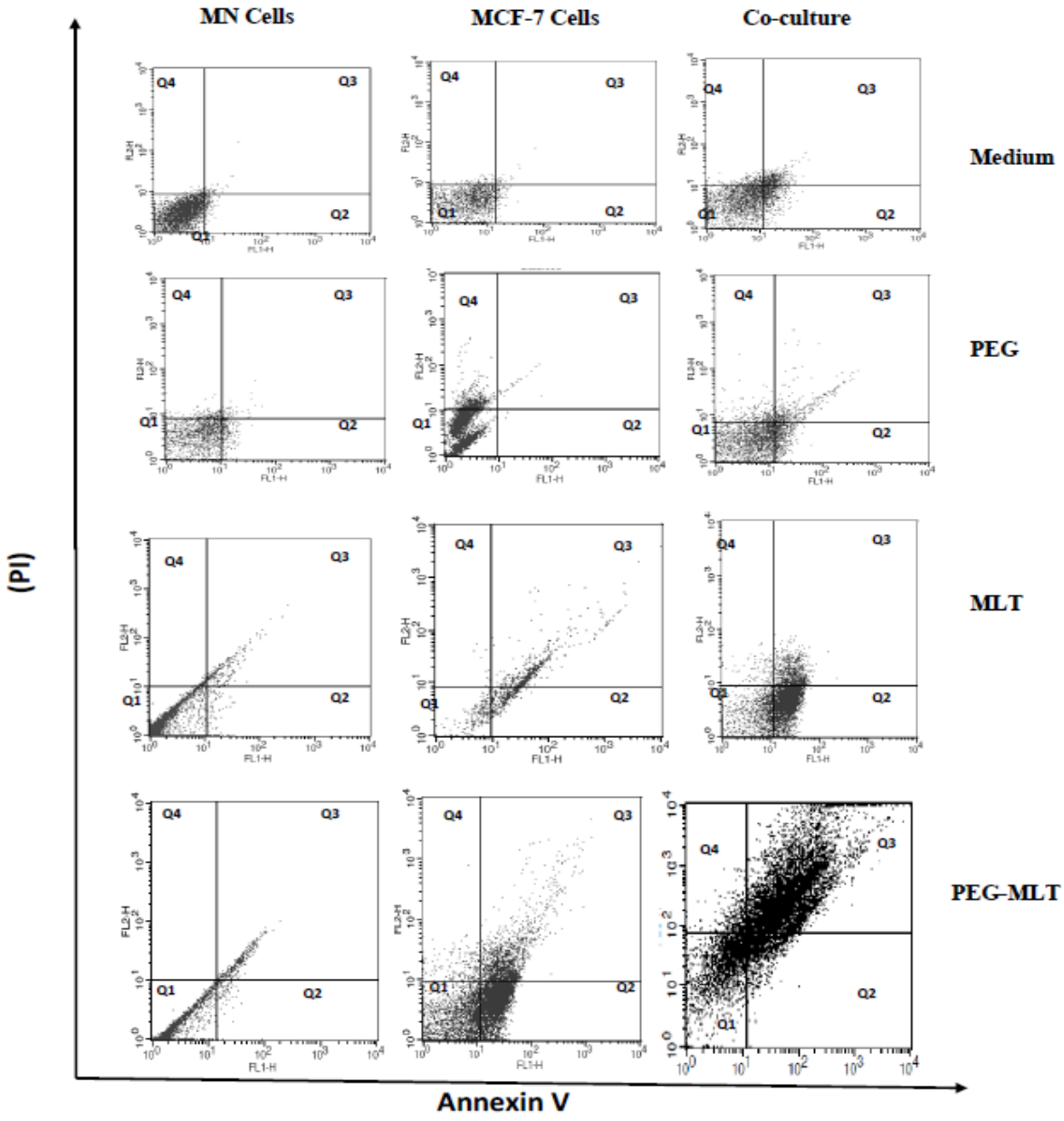

Figure 2. Apoptosis in MCF-7 human breast cancer cells in co-culture of diurnal colostrum MN cells in the presence of PEG microsphere adsorbed with melatonin. Modes of cell death were using flow cytometry with anexinV/PI staining. The summation of the upper-right (Q3) and lower-right (Q2) quadrants is presented as the percentage of total apoptosis. The upper-left (Q4) quadrant is the percentage of necrosis and lower-left

(Q1) quadrant corresponds to viable cells. Data are representative of an experiment with the different treatments.

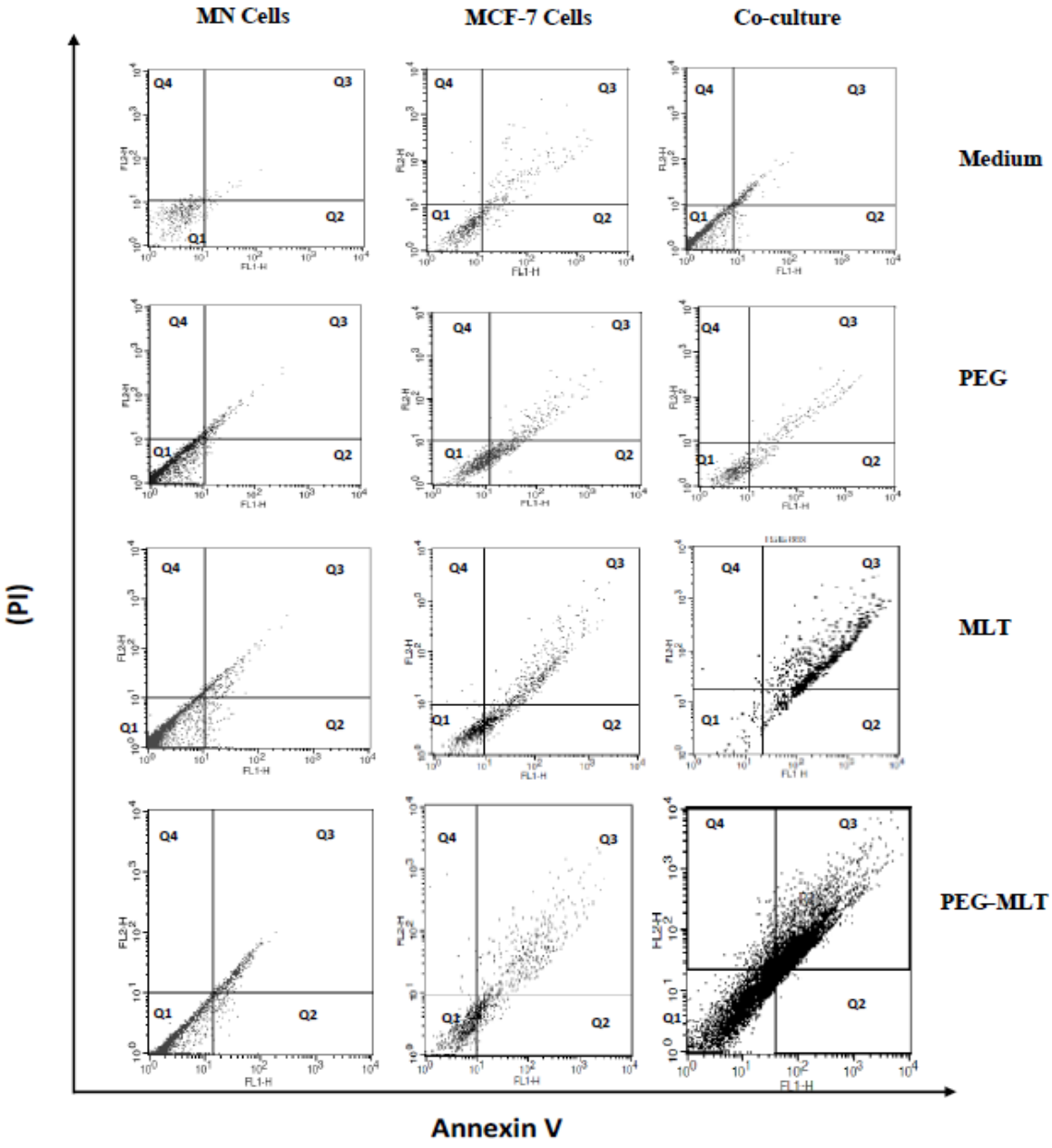

Figure 3. Apoptosis in MCF-7 human breast cancer cells in co-culture of nocturnal colostrum MN cells in the presence of PEG microsphere adsorbed with melatonin. Modes of cell death were using flow cytometry with anexinV/PI staining. The summation of the upper-right (Q3) and lower-right (Q2) quadrants is presented as the percentage of total apoptosis. The upper-left (Q4) quadrant is the percentage of necrosis and lower-left (Q1) quadrant corresponds to viable cells. Data are representative of an experiment with the different treatments.

5.3 Artigo 3: Em elaboração

Modulation chemokines release in culture of colostral mononuclear cells and human breast cancer cells influenced by nanodoses of secretory IgA adsorbed to PEG microspheres

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Word count: 4069

Abstract: 150 words

Manuscript word count: 3778

Number of references: 52

Number of figures: 03

Number of Tables: 04

ABSTRACT

This study investigated the effect of PEG microspheres with nanodoses of secretory immunoglobulin A (SIgA) adsorbed in human colostral mononuclear (MN) cells co-cultured with breast cancer cell lines (MCF-7). MN cells, MCF-7 cells and their co-culture were pre-incubated for 24 h with 100 $ng.mL^{-1}$ SIgA, PEG microspheres (PEG) or 100 $ng.mL^{-1}$ SIgA adsorbed to PEG microspheres (PEG-SIgA). Cells subsets of MN cells, chemokines, viability, intracellular calcium release and apoptosis were determined by flow cytometry. There was an increase in the release of MIG, reduction of IL-8 and MCP-1 in the co-culture of cells incubated with PEG-SIgA. The MN cells in co-cultured with the MCF-7 cells, independent of treatment, showed the highest intracellular Ca^{2+} and apoptosis index. These data suggest that the control of chemokines release and increase intracellular Ca^{2+} and apoptosis in co-culture incubated of PEG-SIgA may be one alternative mechanism involved in protection against the breast tumor.

Keywords: SIgA, PEG microsphere, colostrum, MN cells, breast cancer, MCF-7 cells.

BACKGROUND

Breast cancer is a heterogeneous disease encompassing multiple subgroups with differing molecular signatures, prognoses, and responses to therapies. Antitumor antibodies are promising therapeutics for cancer treatment (1)

In a recent study, it was reported that nanodoses of SIgA from the human colostrum have antitumor effects on human MCF-7 breast cancer cells (2). Human breast milk is particularly rich in secretory IgA (SIgA) antibodies (3-5), which play a protective role against several diseases. The biological activity of SIgA is important because this protein is part of the primary antibody class in human breast milk (4,6), and the presence of large amounts of this protein in secreted breast milk may provide protection against breast tumors in women who breastfed (2)

In addition to SIgA effectors, immune cells may contribute to the efficacy of the antibody as protection against tumor growth. During the past decade, insights have been gained regarding the mechanisms underlying the dynamic interplay between immune cells and tumor progression (7).

Human colostrum contains large amounts of viable leukocytes ($1x10^9$ cells/mL in the first days of lactation). Colostrum cells are constituted by polymorphonuclear (PMN) cells, among them neutrophils, and mononuclear (MN) cells that are lymphocytes and macrophages (6,8). The major mononuclear cells present in the colostrum are macrophages (9). Macrophages differ from blood mononuclear phagocytes by presenting increased motility, number of lysosomes, and SIgA on the surface and at the endocytoplasmatic level (9,10,11), having phagocytic and bactericidal activity, and producing free radicals(4,6,11).

Colostral macrophages may be activated by stimulatory signals generated by milk antibodies, especially during SIgA interaction with its Fc receptor (FcαR or CD89) (8,12). A study has reported that interactions between IgA and FCαR have been identified as a candidate target for tumor therapy, which can lead to tumor cell killing (13). Considering the large amount of SIgA and mononuclear cells in the colostrum, especially macrophages that have receptors for SIgA (8), it is probable that interactions between cells and antibodies may activate tumoricidal mechanisms and contribute to the effects protective against breast cancer that are conferred for the mother during breastfeeding.

However, protein-based drugs, including antibodies, are being developed and may represent an innovative therapeutic. Polyethylene glycol (PEG) microspheres are polymeric particles that have the capacity to adsorb organic compounds and are considered a major drug carrier (14). PEG microspheres are promising because they prevent the degradation of the substances adsorbed and increase their bioavailability in the organism, thereby modulating the immune system (15,16).

The administration of drugs adsorbed to PEG microspheres has been an alternative treatment for a number of diseases (17), including breast cancer, the most common cancer affecting women (18). PEG combined with colostral SIgA has been used to reduce cell viability and induce apoptosis in breast cancer tumor cell lines (MCF-7) (2).

Nevertheless, no studies have linked interactions between SIgA adsorbed to PEG microspheres and colostral mononuclear cells. Its immunomodulatory potential on colostral cells for the initiation of tumor cell killing should be considered to clarify its possible use as a therapy for breast cancer treatment. The aim of this study was to investigate the effect of PEG microspheres with nanodoses of SIgA adsorbed on the

human colostral mononuclear cells co-cultured with breast cancer tumor cell lines (MCF-7).

Methods

Subjects:

This cross-sectional study evaluated 30 mothers clinically healthy at the Health System Program of Barra do Garças, Mato Grosso, Brazil. The volunteers signed an informed consent form before entering the study, which was approved by the local ethics committee Araguaia of the (Protocol Number CAAE: 45102815.3.0000.5587).

Obtaining supernatants from colostrum:

About 8 mL of colostrum from each woman were collected in sterile plastic tubes between 48 and 72 hours postpartum. The samples were centrifuged (160 x g, 4°C) for 10 min, which separated colostrum into three different phases: cell pellet, an intermediate aqueous phase, and a lipid-containing supernatant. The upper fat layer was discarded, the aqueous supernatant stored at -80^{o}C for obtaining purified SIgA and the reserved cells for later analyses.

Colostral Purified SIgA

Human colostrum SIgA was purified from a defatted colostrum pool by affinity chromatography on Cyanogen Bromide-Activated Sepharose-4B (CNBr-Sepharose-4B - Sigma, ST Loius, USA) bound with sheep anti-human α chain as proposed by March et al. (19). To ensure SIgA depletion, fractions eluted from the affinity chromatography column were pooled and passed five times through the same column. Bound SIgA was eluted from the column with 6N glycine – HCL buffer, pH 2.8. The purified preparations were restored to the initial volume. The concentration of SIgA was determined by simple

radial immunodiffusion with a sheep anti-human α chain serum on agarose plates (6) Protein total concentration was available by Lowry Method. The purified SIgA preparation was also tested by immunoelectrophoresis with goat anti-human γ and μ chain antisera. Both IgG and IgM were undetectable in the preparation. The purified SIgA was 4.0 g/L adjusted to a concentration of 100 ng/mL. The aliquots were stocked at -80^{o}C and subsequently used for experiments.

Polyethylene glycol (PEG) microsphere preparation:

The microspheres were obtained from polyethylene glycol (PEG) 6000 using a modification (15) of a previously described protocol (20). Briefly, 20 g of PEG 6000 was resuspended in 100 mL of a 2% sodium sulfate solution in phosphate-buffered saline (PBS) and incubated at 37°C for 45 min. After incubation, the PEG microspheres were diluted 3:1 in PBS and washed twice in PBS (500 x g, 5 min). The PEG microspheres were resuspended in PBS. The formation of the microspheres was thermally induced by subjecting the solution to 95°C for 5 min. For adsorption, the suspensions of PEG microspheres in PBS were incubated with SIgA (Sigma, St. Louis, USA; concentration 100 ng mL-1) at 37°C for 30 minutes. After this period the PEG microspheres with adsorbed SIgA was washed twice in PBS (500 x g, 5 min). The loading efficient of adsorption the PEG microspheres with adsorbed secrotory IgA (SIgA) were verified with fluorescently labeled overnight at room temperature with a solution of Dylight-488 (Pierce Biotechnology, Rockford, USA; 10 μg mL-1) in dimethylformamide at a 100:1 molar ratio of PEG:Dylight and analyzed by fluorescence microscopy (2).

Separation of colostral cells.

Colostrum from each woman was collected in sterile plastic tubes. The samples were centrifuged (160 x g, 4°C) for 10 min. Cells were separated by a Ficoll-Paque gradient

(Pharmacia, Upsala, Sweden). This procedure generated 98% pure mononuclear cell preparations as analyzed by light microscopy (21). Purified macrophages were resuspended independently in serum-free 199 medium at a final concentration of $2x10^6$ cells/ml. After this period, the macrophages were washed twice and used by assay.

MN cells treatment

To explore cells subsets, cell viability (propidium iodide permeability test), apoptosis induction (annexin V staining) and intracellular calcium release (Fluo-3 staining) colostral MN cells were pre-incubated for 24 h with or without 50 µL of SIgA (100 ng/mL final concentration), 50 µL of polyethylene glycol (PEG) microspheres or 50 µL of SIgA adsorbed in PEG microspheres (PEG-IgA) (100 ng/mL final concentration). Next, the cells were diluted in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA), penicillin (20 U/mL) and streptomycin (20 µg/mL) (Sigma, St. Louis, MO, USA) at 37°C for 24 h in a humid atmosphere containing 5% CO_2.

MCF-7 cell culture

MCF-7 human breast cancer cells were obtained from American Type Culture Collection (ATCC, USA). The cells were maintained as monolayer cultures in 75 cm^2 plastic culture flasks in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA), penicillin (20 U/mL) and streptomycin (20 µg/mL) (Sigma, St. Louis, MO, USA) at 37°C in a humid atmosphere containing 5% CO_2. The cells were subcultured every 5±2 days.

MCF-7 Cell treatment

To explore cell viability (propidium iodide permeability test), apoptosis induction (annexin V staining) and intracellular calcium release (Fluo-3 staining), subconfluent

(80%) monolayers of MCF-7 cells were treated with a concentration of trypsin (Sigma, St. Louis, USA) adjusted for $5x10^6$ cells/mL. Then, the trypsinized cells were pre-incubated for 24 h with or without 50 µL of SIgA (100 ng/mL final concentration), 50 µL of polyethylene glycol (PEG) microspheres or 50 µL of SIgA adsorbed in PEG microspheres (PEG-IgA) (100 ng/mL final concentration). Next, the cells were diluted in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA), penicillin (20 U/mL) and streptomycin (20 µg/mL) (Sigma, St. Louis, MO, USA) at 37°C for 24 h in a humid atmosphere containing 5% CO_2.

Co-culture of MN cells and MCF-7 cells

Cell co-culture systems incorporating MN and MCF-7 cells ($5x10^6$ cells/mL -22) were performized using the same protocol describe above.

After this period, the cells co-cultures were washed twice and used for analyzes.

Cell subsets

After the culture with the different treatment the colostrum cells in co-culture or not were stained with 5µL of anti-CD3 PerCP; anti-CD4FITC, anti-CD8PE, anti-CD14FITC for 30 min at room temperature. Cells were washed and resuspended in phosphate-buffered saline (PBS) containing bovine serum albumin (BSA- Sigma, ST Louis, USA; 5mg/mL) for flow cytometry analyses. Isotypic controls (IgG1-FITC, IgG1-PE both from BD Biosciences). A minimum of 10.000 cells were gated by size (FSC) and granularity (SSC) with a flow cytometer (FACSCalibur, BD Biosciences, USA). Data were analyzed using the Flowjo 7.2.5 software. 4.4.5.

Quantification of chemokines in supernatant of culture

Chemokines Interleukin-8 (CXCL8/IL-8), chemokine ligand 5 (CCL5/RANTES), chemokine monokine induced by γ-interferon (CXCL0/MIG), monocyte chemoattractant

protein-1 (CCL2/MCP-1) and protein 10 (CXCL10/IP-10) concentrations in the supernatant of cultures of MN cells, MCF-7 cells and co-culture (MN and MCF-7 cells) were measured by a cytometric bead array (CBA, BD Biosciences, USA) according to the manufacturer procedures. A flow cytometer was used for these analyses (FACSCalibur, BD Biosciences, USA); cytometric graphs were generated using CellQuest (BD Biosciences, USA) software, and the data were obtained using 1.0 FCAP Array (BD) software.

Cellular viability

After 24 h of treatment, the culture medium was removed, and the cells (MN cells or/and MCF-7 cells) were washed two times with phosphate-buffered saline (PBS). The viability test was performed using propidium iodide fluorescence assay.[23] Then, the cells were stained with 10 µL of propidium iodide (PI, 1mg/mL), Triton X-100 (5.5%) and EDTA (110 mM) and incubated for 10 min at room temperature. Unstained cells were used as controls. The fluorescence of the cells was analyzed by flow cytometry (FACSCalibur system; BD, San Jose, USA). The percentage of viable cells was conversely proportional to the geometric mean fluorescence intensity of PI.

Intracellular Ca^{2+} release determination

We analyzed fluorescence staining using a FACSCalibur (BD San Jose, USA) to assess intracellular Ca^{2+} release in MN cells and/or MCF-7 cells. Cells were loaded with the fluorescent radiometric calcium indicator Fluo3-acetoxymethyl (Fluo3-AM; Sigma, St. Louis, USA). Cell suspensions were incubated with 5 µL of Fluo-3 (1 µg/mL) for 30 min at 37°C. After incubation, the MCF-7 cells were washed twice in PBS containing BSA (5 mg/mL; 160 x g, 10 min, 4°C) and then analyzed by flow cytometry (FACSCalibur system; BD, San Jose, USA). Fluo-3 was detected using a 530/30 nm

filter for intracellular Ca^{2+}. The rate of intracellular Ca^{2+} release was expressed as the geometric mean fluorescence intensity of Fluo-3.

Apoptosis assay

Annexin V staining was used to assess apoptosis. Untreated cells were used as negative controls, and cells treated with staurosporine (Sigma, St. Louis, USA - 24) which was used to induce apoptosis, were used as positive controls. The cells (MN cells or/and MCF-7 cells) were resuspended in 500 μL of binding buffer containing 5 μL of annexin V-FITC (Annexin V-FITC Apoptosis Detection Kit, Alexis™, San Diego, USA) and 5 μL of PI and then incubated for 10 min at room temperature. The fluorescence of the cells was analyzed by flow cytometry (FACSCalibur system; BD, San Jose, USA). The obtained data were analyzed using CellQuest software. The cells were classified as follows: viable cells ($annexin^-/PI^-$), early apoptotic cells ($annexin^+/PI^-$), late apoptotic cells ($annexin^+/PI^+$), and necrotic cells ($annexin^-/PI^+$).

Statistical analysis:

An analysis of variance (ANOVA) was used to evaluate cellular proliferation, culture supernatant calcium levels, and the intracellular calcium and apoptosis index of cells in the presence or absence of PEG microspheres with adsorbed SIgA. Statistical significance was considered when $p<0.05$.

RESULTS

The cells treated with SIgA and PEG-SIgA in co-culture with the MCF-7 cells had an increase percentage of $CD3^+$ T cells. There was an increase in CD8+ T cells when these cells were co-cultured with MCF-7 cells and treated with PEG-SIgA (Table 1). It was observed that regardless of treatment or the presence of tumor cells, the highest percentage of mononuclear cells present in the colostrum was macrophages, which represented approximately 80% of cells expressing $CD14^+$ (Table 1).

Chemokines (IL-8, RANTES, MIG, MCP-1 and IP-10) were assessed in the supernatant of the culture of MN cells co-cultured or not with the MCF-7 cells and treated with PEG-SIgA (Table 2). The level of IL-8 was higher in the supernatant of the MN cells than in the supernatant of the MN cells co-cultured with MCF-7 cells and lower in the co-culture treated with PEG-SIgA. RANTES concentrations were similar between groups. The concentration of MIG was higher in the supernatant of the culture of the MN cells treated with SIgA or PEG-SIgA and co-cultured with the MCF-7 cells. The co-culture of MN cells and MCF-7 cells increased the MCP-1 levels, whereas the treatment with PEG-SIgA decreased this chemokine. The co-culture of MN cells and MCF-7 cells reduced the IP-10 concentration (Table 2).

The viability of MN cells is shown in Figure 1A. The MN cells that were not stimulated had a higher viability. The PEG microspheres, SIgA and SIgA adsorbed to the PEG microspheres did not alter cell viability.

The MCF-7 cell viability index (Figure 1B) was higher in the cells without treatment. PEG microspheres did not change cell viability. MCF-7 cells incubated with SIgA or SIgA adsorbed to the PEG microspheres showed lower viability (Figure 1B).

The SIgA and SIgA adsorbed to the PEG microspheres reduced the viability of the co-culture of MN cells and MCF-7 cells (Figure 1C).

Table 3 shows the rate of intracellular Ca^{2+} release in MN cells, MCF-7 cells and co-culture of MN and MCF-7 cells as measured by Fluo-3 fluorescence intensity.

MN cells exhibited low spontaneous intracellular Ca^{2+} release. The PEG microspheres and SIgA did not alter intracellular Ca^{2+} release by the MN cells. When these cells were incubated with SIgA adsorbed to PEG microspheres, they showed a

higher intracellular Ca^{2+} release than non-treated cells and those treated with the PEG microspheres (Table 3).

MCF-7 cells had low spontaneous intracellular Ca^{2+} release. When these cells were incubated with SIgA or SIgA adsorbed to PEG microspheres, they had increased intracellular Ca^{2+} levels (Table 3).

MN cells and MCF-7 cells in co-culture showed higher intracellular Ca^{2+} release when they were incubated with SIgA adsorbed or not adsorbed to PEG microspheres. The highest intracellular Ca^{2+} was observed when the cells were treated with SIgA adsorbed to PEG microspheres (Table 3 - Figure 2).

To evaluate apoptosis induction in MN cells, MCF-7 cells and co-culture (MN and MCF-7 cells), cells were stained with annexin V and analyzed by flow cytometry (Table 4 and Figure 3).

The apoptosis index of MN cells was similar when these cells were incubated with PEG, SIgA or SIgA adsorbed to PEG microspheres (Table 4 -Figure 3A). The PEG increased the necrosis in the MN cells (Table 4).

The apoptosis rate was lower in non-treated MCF-7 cells and those incubated with PEG. The PEG microsphere increased the necrosis in the MCF-7 cells. The apoptosis and necrosis rates were higher in cells that were treated with SIgA or SIgA adsorbed to PEG microsphere (Table 4). The highest apoptosis rates were observed in the MCF-7 cells treated with SIgA adsorbed to PEG microspheres (Table 4-Figure 3 B).

In general, the apoptosis rate in co-cultures of MN cells and MCF-7 cells was higher. The co-culture of cells with PEG or SIgA increased the necrosis rates. The

highest apoptosis index was observed in co-cultures of cells incubated with SIgA adsorbed to PEG microspheres (Table 4-Figure 3 C).

DISCUSSION

The combination of antibodies and polymeric matrices has been developed as an alternative therapy for a number of diseases, including cancer. In the present study, SIgA adsorbed to PEG microspheres exhibited strong antitumor activity against MCF-7 human breast cancer cells, especially when in co-culture with colostral MN cells.

The modified delivery system is capable of maintaining an adequate period until the protein reaches its required site of action and then releases it in a controlled fashion (25). PEG microspheres act by extending the time of the relative bioavailability of the drug compared with that of the free drug and potentiating the pharmacological action (17,26,27).

Mucosal immunity is of great importance as a natural barrier, and SIgA acts as the first line of defense against pathogens and other potentially harmful agents. Human breast milk is particularly rich in secretory IgA (SIgA), and interactions of this antibody with cells of the secretion itself are extremely important to provide protection for various diseases (4,8,11). In this work, approximately 80% of the colostrum cells obtained were macrophages. Several factors influence the distribution of cell types in milk. The study of cells from milk provides a good model for cancer research (28), especially breast cancer (7). Although macrophages represent a major component of immune infiltrate in breast cancer and can contribute to the progression and dissemination of tumors, studies suggest that these cells can be reprogrammed to act as a potent antitumor immune response (29). It should be considered that colostrum macrophages are cells that present morphofunctional differences in relation to other types of monocytes and macrophages

present in other tissues which can, independently of reprogramming, exert anti-tumor effects on the breast cancer.

Interestingly, the PEG-SIgA could increase T cells expressing $CD8^+$ when in co-culture with MCF-7 cells. CD8+ T lymphocytes are an important component of the tissue microenvironment and can recognize and eliminate breast tumor cells (30). Here, the increase of CD8+ T lymphocytes by PEG-SIgA probably should be due to an activation and proliferation of these cells. More studies should be conducted to investigate the effects of PEG-SIgA on other isolated myeloid cells to clarify the antitumor mechanisms that involve the cells of the colostrum.

The diversity of the immunostimulatory molecules has been associated with antitumor effectors (31). The chemoattractive properties of chemokines on different leukocyte subpopulations and its effects in the development of antitumor immune responses have been shown (32). Chemokines are a superfamily of low molecular weight chemotactic cytokines that present effects on cells (33). In this study, we showed that in co-culture of MN cells and MCF-7 cells in the presence of PEG-SIgA, there was an increase in the release of MIG and reduction in the concentrations of IL-8 and MCP-1. Interestingly, in the absence of PEG-SIgA, there was an increase in IL-8 and MCP-1 with lower concentrations of IP-10.

Antitumor activity of MIG and IP-10 has been reported in various tumor models (33,34). Evidence suggests that these chemokines are also key to tumor inhibition mediated by other cytokines and chemokines (35,36,37). However, MCP-1 and IL-8 have been associated with the progression of tumors (38). In this study, we showed the control of chemokine release in the presence of PEG-SIgA probably can contribute to the antitumor activity.

On the other hand, the anticancer effects attributed to SIgA were demonstrated by *in vitro* studies of human breast cancer cell lines (2). The propidium iodide fluorescence assay (PIA) characterizes *in vitro* growth of human tumor cell lines due to their ability to penetrate only damaged cell membranes (23,39). Applying this method, we found that SIgA decreased human MCF-7 cell growth and that this effect was more pronounced when these cells were co-cultured with colostral MN cells treated with SIgA adsorbed to PEG microspheres. The data suggest that microspheres modify SIgA release, activating the MN cells and improving its oncostatic effects.

However, the balance between a protective secretory immunity response and the degree to which the cellular and soluble components are present in human milk should be to promote reducing breast cancer, which depends on factors that can be regulated systematically by an immune response (2). Studies have suggested that the low IgA production in patients with cancer may be an additional risk factor for tumor development (40). Due to the complicated pathogenesis of cancer and versatile refractory of tumor cells, combinatorial strategies are more favored in cancer therapies. Exogenous monoclonal antibodies to neoantigens have reduced the growth of tumors by various mechanisms, among these being apoptosis (41,42).

Apoptosis is intensified in several diseases, including cancer (43,44), and is essential in anticancer chemotherapy since it is promoted by the drugs used in this treatment (45,46,47). Thus, the identifying substance that potentiates apoptosis in cancer cells is of great interest, especially when searching for new strategies for the prevention and treatment of breast cancer using natural proteins with potential anticancer properties (2), and their mechanisms of action are promising as a therapy for breast cancer (48).

Here, SIgA from the human colostrum adsorbed to PEG microspheres stimulated the apoptosis of MCF-7 cells in co-culture with MN cells. The increase in apoptosis was also accompanied by an increase in intracellular calcium levels. Interestingly, adsorption of SIgA to the PEG microspheres was able to reduce the necrosis, suggesting that the SIgA-PEG can modify the effects of the antibody on mechanisms of cell death both in tumor cells and in colostrum cells.

Studies have linked the apoptosis rate that increased in MCF-7 cells with modifications in intracellular Ca^{2+} release (49,50), being that the control of intracellular calcium release is essential for cellular activity tests in response to modulating agents (51). It should be considered that there is a high concentration of phagocytes in the secretion and that the SIgA-cell interactions occurs via its Fc alpha receptor (CD89 – 6,11); these interactions are responsible for initiating different killing mechanisms (13) and induction of apoptosis in phagocytes (52). Further studies should be performed in order to verify the possible involvement of the Fc alpha receptor during cell interactions and PEG-SIgA interactions.

In conclusion, the effects of nanodoses PEG-SIgA favoring the release of chemokines with antitumor activity, reducing viability and increasing the influx of intracellular calcium and the indices of apoptosis in co-culture of colostral MN cells and MCF-7 may be one of the mechanisms involved in protection against the breast tumor and a possible therapeutic alternative for treating this disease. In addition, the interaction of these soluble and cellular components present in colostrum may be responsible for mechanisms that reinforce the hypothesis that breastfeeding reduce the risk of breast cancer.

ACKNOWLEDGMENTS

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq Nº: 447218/2014-0; Nº: 308600/2015-0), Brazil.

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Table 1. Surface phenotypes of colostral mononuclear (MN) cells. Percentage of T cells ($CD3^+$), T helper cells ($CD4^+$), T cytotoxic cells ($CD8^+$) and macrophages ($CD14^+$) in the human colostrum after 24 hours in culture.

Culture	**Subsets cells (%)**			
	$CD3^+$	$CD4^+$	$CD8^+$	$CD14^+$
MN cell	15.7±5.6	8.2±1.6	4.4±2.9	80.3±7.8
MN cell+ PEG	14.6±2.5	8.1±2.4	6.2±3.4	81.5±9.7
MN cell+MCF-7	14.5±2.	9.0±2.6	7.2±3.8	79.9±5.9
MN cell+MCF-7+SIgA	17.5±1.8*	7.1±3.9	6.5±2.6	82.5±8.7
MN cell+MCF-7+PEG-SIgA	18.8±1.6*	8.3±2.7	9.3±1.2*	80.5±7.2

Notes: Polyethylene glycol (PEG) microspheres; secretory immunoglobulin A (SIgA); secretory immunoglobulin A adsorbed to PEG microspheres (PEG-SIgA). $P<0.05$ between treatments (PEG, SIgA and PEG-SIgA) considering the same kind of subsets of cells.

Table 2. Chemokine concentrations in the supernatant of cultures of colostral MN cells treated with SIgA and PEG-SIgA, co-cultured but not with MCF-7 cells after 24 hours in culture.

Culture	**Chemokines (pg/mL)**				
	IL-8	RANTES	MIG	MCP-1	IP-10
MN cell	16.7±3.2	78.5±19.1	79.8±21.3	41.8±25.4	72.8±17.8
MN cell+ PEG	16.2±2.9	67.6±36.24	71.7±9.4	34.8±5.8	69.4±18.1
MN cell+SIgA	26.6±3.2*	43.4±6.3	66.2±9.6	46.2±25.0	101.6±52.9
MN cell+ PEG-SIgA	9.2±2.8	42.2±27.6	93.2±10.3	55.2±5.8	59.6±4.5
MN cell+MCF-7	26.4±7.4*	79.9±6.5	36.6±3.9	102.2±5.7 *	46.6±18.6*
MN cell+MCF-7+SIgA	10.5±1.8	40.4±7.9	95.4±6.9*	36.9±10.4	48.7±18.0
MN cell+MCF-7+PEG-SIgA	4.9±1.3*	71.3±12.0	101.9±14.4*	18.4±8.9*	62.8±8.3

Notes: The results represent the mean ± SD of six experiments. Polyethylene glycol (PEG) microspheres; secretory immunoglobulin A (SIgA); secretory immunoglobulin A adsorbed to PEG microspheres (PEG-SIgA). *$p<0.05$ treated cells compared with non-treated cells (PBS); $^{+}P<0.05$ between treatments (PEG, SIgA and PEG-SIgA).

Table 3. Release of intracellular Ca^{2+} by MN cells, MCF-7 cells and co-cultures (MN cells and MCF-7 cells) in the presence of SIgA adsorbed to PEG microspheres.

	MN Phagocytes	MCF- Cells	Coculture
PBS	2.5±0.7	5.6±1.6	16.1±5.6$^{\#}$
PEG	2.9±1.2	6.5±1.5	13.9±5.6$^{\#}$
SIgA	5.7±2.2	13.9±1.1*$^{\#}$	19.9±0.6*$^{\#}$
PEG- SIgA	15.5±2.2*$^{+}$	16.1±2.3*	29.5±5.8*$^{+\#}$

Notes: Intracellular Ca^{2+} release is represented by mean fluorescence intensity as determined by flow cytometry. The results represent the mean ± SD of six experiments. Phosphate buffered saline (PBS); Polyethylene glycol (PEG) microspheres; secretory immunoglobulin A (SIgA); secretory immunoglobulin A adsorbed to PEG microspheres (PEG-SIgA). *$p<0.05$ treated cells compared with non-treated cells (PBS); $^{+}P<0.05$ between treatments (PEG, SIgA and PEG-SIgA); $^{\#}P<0.05$ comparing MN cells, MCF-7 cells and co-cultures (MN cells and MCF-7 cells).

Table 4**.** Apoptosis (%) and necrosis (%)of colostral Mononuclear (MN) Cells, MCF-7 Cells and co-culture of colostral MN Cells and MCF-7 Cells in the presence of SIgA adsorbed to PEG microspheres.

	Viables Q1	**Apoptosis (Q2+Q3)**	**Necrosis Q4**
MN	91.8±0.9	6.7±1.7	1.5±0.3
MN +PEG	90.0±2.5	5.9±2.1	4.1±1.0*
MN +SIgA	89.7±0.9	7.4± 1.3	1.9±0.1
MN+PEG- SIgA	89.1±1.3	9.6 ±2.3	$2.2\pm0.5^{\#}$
MCF-7	93.1±0.8	3.1±0.6	3.8±1.8
MCF-7+PEG	80.2±0.6*	4.9±0.5	14.5±0.7*
MCF-7+SIgA	49.7±8.3*	41.2± 10.3*	9.1±0.9*
MCF-7+PEG- SIgA	$31.3\pm4.5^{*\#+}$	$55.8\pm7.1^{*\#+}$	12.9±1.7*
MN+ MCF-7	69.8±9.8	24.4±5.2	5.8±1.2
MN+ MCF-7+PEG	62.6±3.2	20.7±5.5	16.7±2.3*
MN+ MCF-7+SIgA	41.3±5.9*	45.9± 12.8*	12.8±2.8*
MN+ MCF-7+PEG- SIgA	$24.0\pm9.5^{*\#}$	$68.5\pm12.5^{*\#+}$	$7.5\pm1.3^{\#+}$

Notes: The results represent the mean ± SD of five experiments. Polyethylene glycol (PEG) microspheres; secretory immunoglobulin A (SIgA); secretory immunoglobulin A adsorbed to PEG microspheres (PEG-SIgA). Q1: Viable cells ($annexin^-/PI^-$); Q2 ($annexin^+/PI^-$) and Q3 ($annexin^+/PI^+$): total apoptotic cells; Q4: necrotic cells ($annexin^-/PI^+$). *$p<0.05$: treated cells compared with non-treated cells; #$p<0.05$: comparing the different treatments (PEG-SIgA with PEG microspheres); $^+p<0.05$: comparing the different treatments (SIgA and PEG-SIgA)

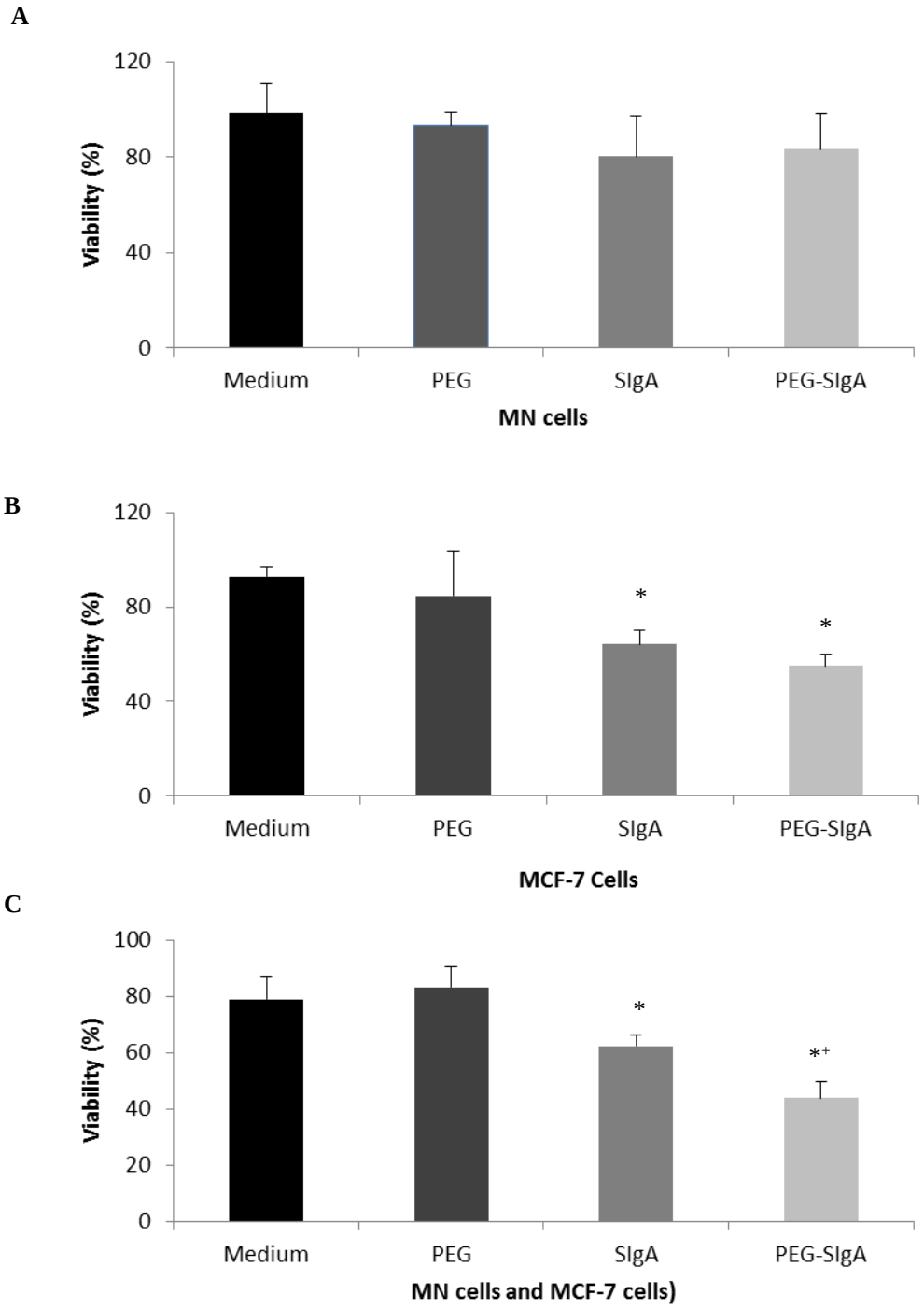

Figure 1. Viability Index of colostral Mononuclear (MN) Cells (A), MCF-7 Cells (B) and co-culture of colostral MN Cells and MCF-7 Cells (C). Cells treated with Polyetilenoglycol (PEG) microspheres, with secretory immunoglobulin A (SIgA) and secretory immunoglobulin A adsorbedby thePolyethylene Glycol microspheres(PEG-SIgA). The results represent the median of the standard of six MN cells from different individuals and six experiments with MCF-7 cells. *$p<0.05$ treated cells compared with non-treated cells (PBS); $^{+}P<0.05$ between treatments (SIgA and PEG-SIgA).

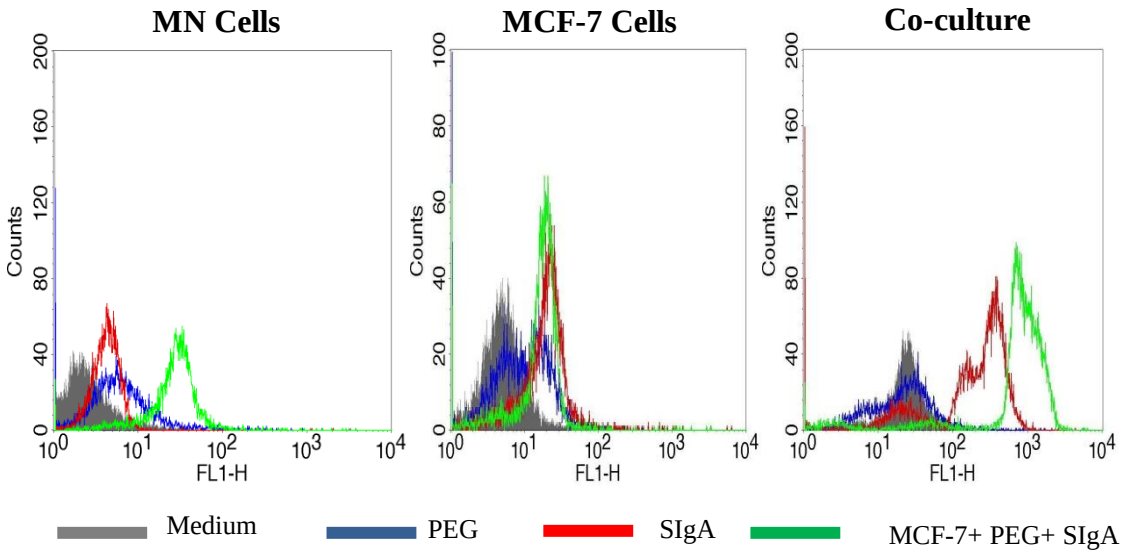

Figure 2. Intracellular Ca^{2+} release by MN cells, MCF-7 human breast cancer cells and Co-culture treated with SIgA adsorbed to polyethylene glycol (PEG) microspheres. Cells were loaded with the fluorescent radiometric calcium indicator Fluo3-Acetoxymethyl (Fluo3-AM– Sigma ST Louis, USA) and Ca^{2+} release was determined by an immunofluorescence assay and flow cytometry (FACScalibur, Becton Dickinson, USA). Polyethylene glycol (PEG) microspheres; secretory immunoglobulin A (SIgA); secretory immunoglobulin A adsorbed to PEG microspheres (PEG-SIgA). Flow cytometric histogram showing the intensities of green fluorescence (FL1-H) on the x-axes and the cell counts on the y-axes.

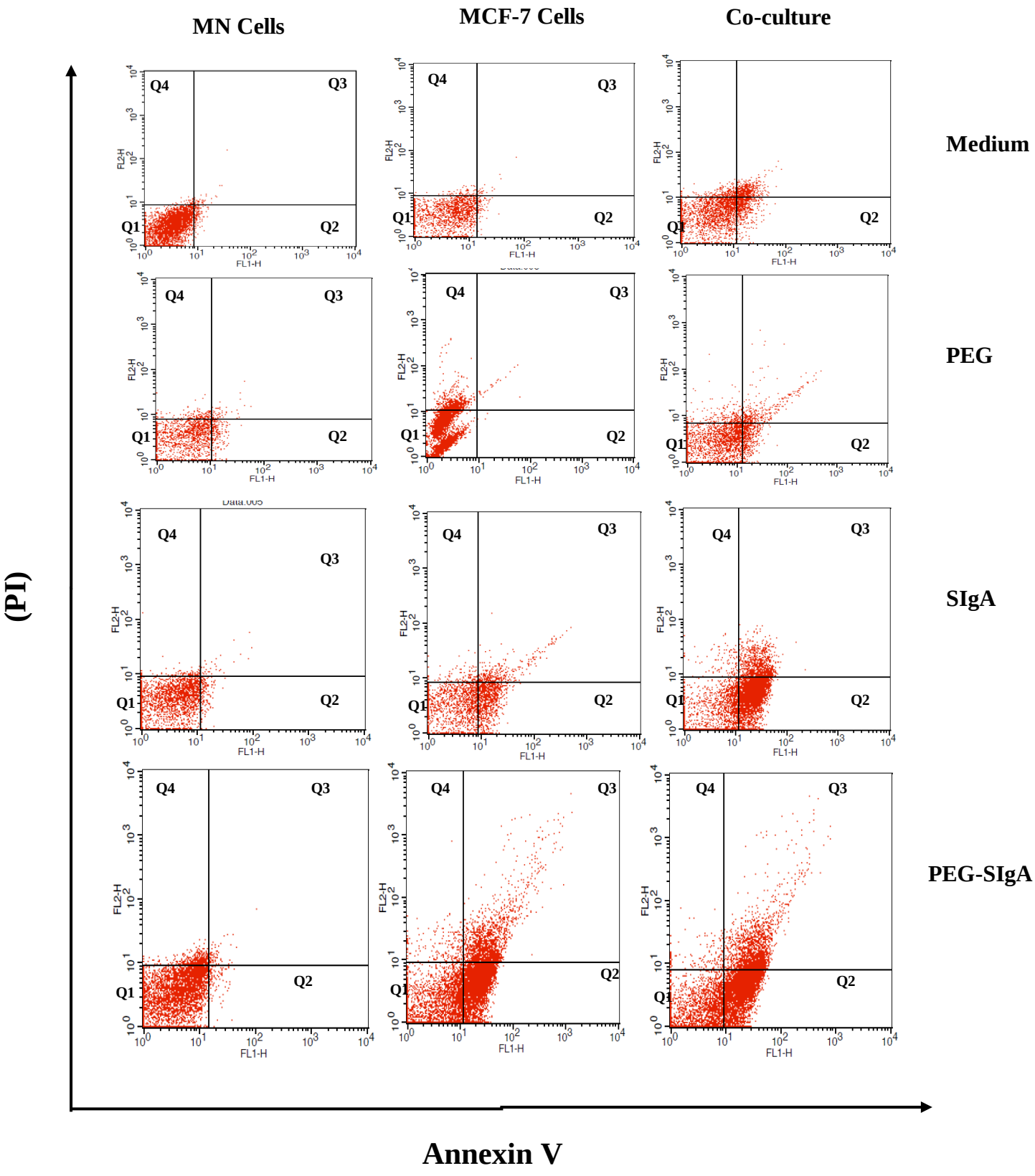

Figure 3. PEG microsphere adsorbed with SIgA induces apoptosis in MCF-7 human breast cancer cells in co-culture of colostral MN cells. Modes of cell death were using flow cytometry with anexinV/PI staining. The summation of the upper-right (Q3) and lower-right (Q2) quadrants is presented as the percentage of total apoptosis. The upper-

left (Q4) quadrant is the percentage of necrosis and lower-left (Q1) quadrant corresponds to viable cells. Data are representative of an experiment with the different treatments.

6. CONCLUSÕES

-Nanofrações de PEG-SIgA foram capazes de aumentar a expressão de células TC8+ quando em co-cultura com células MCF-7, o que provavelmente pode ser devido a uma ativação e proliferação dessas células.

-Na quantificação das quimiocinas no sobrenadante da cultura revelou-se que houve aumento na liberação de MIG e redução nas concentrações de IL-8 e MCP-1 em co-cultura de células MN e células MCF-7 na presença de PEG-SIgA.

-Nanofrações de SIgA adsorvida ou não à microesfera de PEG foram capazes de reduzir a viabilidade da co-cultura de células MN e células MCF-7.

-A liberação de cálcio intracelular e apoptose foi maior quando a co-cultura foi incubada com SIgA adsorvida a microesferas de PEG.

-A melatonina adsorvida em microesferas de PEG foi capaz de aumentar, em co-cultura com as células MCF-7, a liberação de superóxido e SOD no período noturno. Já as células MCF-7 tratadas com MLT adsorvida em microesferas de PEG mostraram aumento de espécie reativa durante o dia e aumento do efeito antioxidante durante a noite.

-A MLT foi capaz de induzir na co-cultura o aumento da liberação de cálcio intracelular e apoptose no período noturno.

Portanto, a interação desses componentes solúveis e celulares presentes no colostro pode ser responsável por mecanismos que reforçam a hipótese de que a amamentação reduz o risco de câncer de mama. E as variações cronobiológicas podem atuar como agentes antitumorais durante diferentes fases do dia.

7. CONSIDEREÇÕES FINAIS

Neste trabalho demostramos a importância da amamentação na prevenção do câncer de mama em mulheres, e comprovamos o quanto os constituintes do leite materno podem influenciar na defesa contra células tumorais. Assim a associação de imunomoduladores à sistemas de liberação modificada, podem representar uma valiosa alternativa para imunoterapia em pacientes com câncer de mama.

A amamenteção traz inúmeros benefícios à mãe e aos recém-nascidos, por isso é importantíssimo o incentivo à prática da amamentação, bem como a implantação de bancos de leite para que esse líquido tão preciso e rico possa alcançar aos bebês que não tem a possibilidade de o ter pela amamentação.

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APÊNDICE

Universidade Federal de Mato Grosso
Campus Universitário do Araguaia
Instituto de Ciências Biológicas e da Saúde
Programa de Pós-graduação em Imunologia e Parasitologia Básicas e Aplicadas

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Projeto: LIBERAÇÃO MODIFICADA DE AGENTES ATIVADORES DE CÉLULAS DE COLOSTRO PARA AÇÃO EM CÉLULAS TUMORAIS DE MAMA.

Pesquisadores e instituições envolvidas: Profa. Dra. Adenilda Cristina Honório França - Instituto Universitário do Araguaia – Universidade Federal de Mato Grosso (UFMT). Rubian Trindade da Silva Fernandes- Instituto Universitário do Araguaia – Universidade Federal de Mato Grosso (UFMT).
Objetivo principal: O objetivo deste projeto é avaliar a liberação modificada de agentes ativadores de células de colostro para ação em células tumorais de mama.
Procedimentos: Coleta de leite humano (colostro) excedente, cerca de 8ml, por ordenha manual (retirada manual do leite, através de massagem e expressão das mamas).
Possíveis riscos e desconforto: não há riscos previsíveis, mas poderá haver certo desconforto, resultante da expressão manual das mamas, apesar desta ser realizado com técnica adequada e de maneira cuidadosa.
Benefícios previstos: Para as atuais participantes do estudo: incentivo à amamentação, assistência e orientações sobre a amamentação e melhorar os conhecimentos sobre os mecanismos de defesa imunológica presentes no leite, que são passados aos seus filhos durante a amamentação. Para as gestantes futuras, possibilidade de novos conhecimentos sobre os benefícios do leite materno e do processo de amamentação.
Eu....., fui informada dos objetivos, procedimentos, riscos e benefícios desta pesquisa, descritos acima. Entendo que terei garantia de confidencialidade, ou seja, que apenas os resultados dos exames realizados com o leite materno serão divulgados e ninguém, além dos pesquisadores, terá acesso aos nomes dos participantes desta pesquisa. Entendo também, que tenho direito de receber, sempre que desejar, outras informações sobre o estudo, entrando em contato com a pesquisadora
(Dra. Adenilda). Fui informada ainda, que a minha participação é voluntária e que, se eu preferir não participar ou deixar de participar deste estudo em qualquer momento, isso NÃO influenciará no meu atendimento junto ao Hospital. Compreendendo tudo o que me foi explicado sobre o estudo e, estando de acordo em participar, assino embaixo.
Assinatura do participante (ou do responsável, se menor):

.....

Assinatura do pesquisador principal:

.....
Profa. Dra. Adenilda Cristina Honório França. Rodovia BR-070, Km 5. Barra do Garças - Mato Grosso. CEP: 78600-000. adenilda@ufmt.br

ANEXO 1

UNIVERSIDADE FEDERAL DE MATO GROSSO - CAMPUS DO ARAGUAIA

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: LIBERAÇÃO MODIFICADA DE AGENTES ATIVADORES DE CÉLULAS DE COLOSTRO PARA AÇÃO EM CÉLULAS TUMORAIS DE MAMA.

Pesquisador: Adenilda Cristina Honorio França

Área Temática:

Versão: 1

CAAE: 45102815.3.0000.5587

Instituição Proponente: Universidade Federal de Mato Grosso

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.064.829

Data da Relatoria: 15/05/2015

Apresentação do Projeto:

O projeto está bem apresentado e está de acordo com as normas do comitê.

Objetivo da Pesquisa:

O objetivo desta projeto é avaliar a liberação modificada de agentes ativadores de células de colostro para ação em células tumorais de mama.

Avaliação dos Riscos e Benefícios:

Considerando que será coletado manualmente o excedente do leite materno,os riscos são mínimos.

Comentários e Considerações sobre a Pesquisa:

Trata-se de um projeto que pretende identificar alterações tumorais e o papel dos componentes imunes presentes no leite humano sobre células tumorais. Este projeto se
torna relevante, uma vez que podem atuar na prevenção e na proposição de novas terapias para o tratamento da doença. O projeto atual pretende
aprofundar em novos aspectos relacionados às respostas imunológicas para tumores, bem como avaliar os efeitos dos componentes do leite materno sobre o câncer.

Considerações sobre os Termos de apresentação obrigatória:

Adequado

Endereço: Rod. MT100 Km 3,5 ICBS
Bairro: Campus do Araguaia CEP: 78.698-000
UF: MT Município: PONTAL DO ARAGUAIA
Telefone: (66)3410-1121 E-mail: mariaaugusta@yahoo.com.br

UNIVERSIDADE FEDERAL DE MATO GROSSO - CAMPUS DO ARAGUAIA

Plataforma Brasil

Continuação do Parecer: 1.894.820

Recomendações:

nenhuma

Conclusões ou Pendências e Lista de Inadequações:

Aprovado

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Projeto aprovado.

PONTAL DO ARAGUAIA, 15 de Maio de 2013

Assinado por:
Marly Augusta Lopes de Magalhães
(Coordenador)

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