Programa de Pós-graduação em Ciências e Técnicas Nucleares – PPGCTN – Departamento de Engenharia Nuclear
Escola de Engenharia
Universidade Federal de Minas Gerais – UFMG

Papel do Extrato da Própolis *Tetragona clavipes* na modulação de interleucinas, fator de crescimento, e na viabilidade *in vitro* de células de adenocarcinoma de mama e mononucleadas do sangue periférico humano, expostas a cobalto-60

Patrícia Lima Falcão Valença

Belo Horizonte
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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS E TÉCNICAS NUCLEARES

FOLHA DE APROVAÇÃO

Papel do Extrato da Propolis Tetragona clavipes na modulação de interleucinas, fator de crescimento, e na viabilidade in vitro de células de adenocarcinoma de mama e mononucleadas do sangue periférico humano, expostas a cobalto-60

PATRICIA LIMA FALCÃO VALENÇA

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RESUMO

O câncer de mama é uma doença agressiva cuja incidência e mortalidade tem aumentado nos últimos anos. Por esse motivo tem despertado preocupação por parte das políticas de saúde pública, sendo alvo de pesquisas por parte da comunidade científica. Dentre os tratamentos para o câncer de mama tem-se a radioterapia, que se emprega radiação ionizante, no entanto, com limitações devido seus efeitos tóxicos nos tecidos normais. O desenvolvimento de drogas de origem vegetal associado ao tratamento de radioterapia é promissor, uma vez que estas substâncias modificam os efeitos deletérios das radiações minimizando seus danos no indivíduo. O presente estudo reúne e documenta a trajetória de estudos que envolveram as linhagens celulares de adenocarcinoma (MDAMB-231), de células mononucleadas de sangue periférico humano (PBMC - Peripheral Blood Mononuclear Cells) e da Própolis *Tetragona clavipes*, de interesse, no Núcleo de Radiação Ionizante (NRI), em abordagens *in vitro*. O principal objetivo foi avaliar o efeito do Extrato Aquoso da Própolis (EAP) sobre a viabilidade de células da linhagem de adenocarcinoma de mama e de células mononucleares do sangue periférico humano, bem como investigar a modulação de citocinas e fatores de crescimento induzidas por radiação. É feito uma análise do papel das citocinas nos processos na modulação da resposta inflamatória/imune ao tumor, considerando que tais marcadores provavelmente poderão estar associados tanto ao potencial clonogênico dessa linhagem. As linhagens celulares foram irradiadas com radiação de baixa LET (Linear Energy Transfer) de acordo com a cinética de doses pré-determinadas (2 e 5 Gy) e suplementadas com EAP nas concentrações de 1% e 10%. As coletas pós-irradiação foram realizadas nos tempos pré-estabelecidos de 24, 48 e 72 horas. O mesmo procedimento foi adotado, e alíquotas em mesma cinética de tempo foi analisada tanto para IL-6 quanto para TGF-beta. O presente estudo mostrou que as linhagens de MDAMB-231 e de PBMCs apresentaram comportamentos diferenciados *in vitro*. A radiação ionizante produziu efeitos de diminuição significativa nos parâmetros de viabilidade celular em PBMCs, enquanto no grupo de células suplementadas com EAP em adição à radiação, os danos causados foram minimizados. Enquanto, nas células de adenocarcinoma de mama MDAMB-231 foi observada uma redução no nível de sobrevivência dessas células quando submetidas à radiação, ao passo que esse efeito foi potencializado quando a cultura de células foi suplementada com extrato aquoso de própolis. A suplementação com extrato de própolis na presença

de radiação ofereceu uma proteção estatisticamente mensurável contra danos às células PBMCs. Além disso, o extrato de própolis parece potencializar a apoptose celular de células de adenocarcinoma de mama radio resistente. Os dados obtidos através desta investigação podem servir como subsídio para a utilização do extrato de própolis como adjuvante ao os efeitos do tratamento radioterápico em pacientes com câncer de mama. As respostas da ação da própolis em nível molecular, através dos mecanismos de ação de fatores inflamatórios na célula, foram apresentadas, e delineiam um caminho para novas pesquisas. O caminho de sinalização de diversas citocinas, modulada por substâncias naturais, pode alterar o microambiente do tumor contribuindo para a supressão de seu desenvolvimento, migração e metástase.

Palavras chaves: câncer, mama, radiação e própolis

ABSTRACTS

Breast cancer is an aggressive disease whose incidence and mortality has increased in recent years. For this reason, it has aroused concern on the part of public health policies, being the target of research by the scientific community in the world. Among the treatments for breast cancer is radiotherapy, which uses ionizing radiation, however, with limitations due to its toxic effects on normal tissues. The development of drugs of plant origin associated with the treatment of radiotherapy is promising, since these substances modify the effects of radiation, minimizing its damage to the individual. The present study documents the trajectory of the studies that involved the adenocarcinoma cell lines (MDAMB-231) and human peripheral blood (PBMC) in the Ionizing Radiation Nucleus (NRI), in *in vitro* approaches and, therefore, the main objective was to evaluate *in vitro* effect of Aqueous Propolis Extract (EAP) on the cell viability of cells of the breast adenocarcinoma lineage and on the cell viability of human peripheral blood mononuclear cells The cell lines were irradiated with low LET (Linear Energy Transfer) radiation according to the kinetics of predetermined doses (2 and 5 Gy) and supplemented with EAP in concentrations of 1% and 10%. The post-irradiation collections were performed at the pre-established times of 24, 48 and 72 hours. The present study showed that the strains of MDAMB-231 and PBMCs showed different behaviors *in vitro*, and that ionizing radiation produced effects of significant decrease in the parameters of cell viability in PBMCs, while in the group of cells supplemented with EAP in addition to radiation, the damage caused has been minimized. With respect to MDAMB-231 breast adenocarcinoma cells, a reduction in the level of survival of these cells was observed when subjected to radiation, whereas this effect was enhanced when the cell culture was supplemented with aqueous extract of propolis. Supplementation with propolis extract in the treatment of radiotherapy offered quite measurable protection against damage to PBMCs cells. In addition, the propolis extract appears to potentiate cell apoptosis of radio-resistant breast adenocarcinoma cells. The data obtained through this investigation can serve as a subsidy for the use of propolis extract as an adjunct to the effects of radiotherapy treatment in patients with breast cancer.

Key words: cancer, breast, radiation and propolis

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LISTA DE ABREVIATURAS

AEM - Auto Exame de Mamas

AET – Brometo de 2-aminoetilisotiuréia

CAPE – éster fenetílico ácido cafeíco

CO_2 – Dióxido de carbono

ECM - Exame Clínico de Mamas

ELISA – (Enzyme-linked Immunosorbent Assay) – Ensaio Imunoenzimático

Gy – Gray

IgA - Imunoglobulina A

IgG - Imunoglobulina G

IgM - Imunoglobulina M

INCA – Instituto Nacional do Câncer

LET - Linear Energy Transfer

MTT – Sal brometo de 3-(4,5-dimetil-tiazol-2-il)-2,5-difenil-tetrazólio

MDA- 231 Célula de adenocarcinoma mamário humano

NK - Natural Killer

PBMC - Peripheral Blood Mononuclear Cells – Células mononucleares do sangue periférico

ROS - Species oxigen reactives

RPMI-1640 – Meio desenvolvido pelo Instituto Roswell Park Memorial, utilizado para cultura de leucócitos

RT - Radioterapia

SBF – Soro fetal bovino

UFMG – Universidade Federal de Minas Gerais

UFAM- Universiade Federal do Amazonas

UNSCEAR – United Nations Scientific Committee on the Effects of Atomic Radiation

UV- ultravioleta

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CAPÍTULO 1

INTRODUÇÃO

1.1- Tema

A investigação do papel de produtos naturais, como a própolis, em estudos *in vitro* e *in vivo*, quando finalizados, poderão suportar os tratamentos radioterápicos atuais, e provavelmente diminuir o tempo de tratamento, elevando a sua eficiência, uma vez que o efeito radioprotetor da própolis parece contribuir para a eficiência do sistema imunológico em resposta ao fracionamento de doses de radiação prescritas nos planejamentos radioterápicos em diversos tipos de tumores. (Ebeid *et al.*, 2016)

Considerando a relevância do tema, a abordagem do uso de produtos naturais nos tratamentos de doenças graves na atualidade, bem como resultados preliminares do grupo de pesquisa Núcleo de Radiações Ionizantes – NRI/EE/UFMG envolvendo a Própolis e radiação, foi proposta uma abordagem *in vitro* dos efeitos do extrato da própolis sobre células humanas associadas ao sistema imune, como base as células mononucleares do sangue periférico (PBMC), e de células da linhagem de adenocarcinoma de mama MDAMB-231, irradiadas com cobalto-60, em doses pré-estabelecidas.

O câncer é o principal problema de saúde pública no mundo e atualmente encontra-se entre as quatro principais causas de morte prematura (antes dos 70 anos de idade) na maioria dos países. O aumento da incidência e mortalidade por câncer no mundo pode ser explicado, em parte, pelo envelhecimento, pelo crescimento

populacional, como também pela mudança na distribuição e na prevalência dos fatores de risco de câncer, especialmente aos associados ao desenvolvimento socioeconômico. Observa-se uma transição dos principais tipos de câncer observados nos países em desenvolvimento, com um declínio dos tipos de câncer associados a infecções e o aumento daqueles associados à melhoria das condições socioeconômicas com a incorporação de hábitos e atitudes associados à urbanização (sedentarismo, alimentação inadequada, entre outros. (INCA, 2020).

No escopo das ações de controle das doenças não transmissíveis, a Epidemiologia do câncer fornece os subsídios para que os gestores monitorem e organizem as ações para o controle da doença, bem como o direcionamento da pesquisa (INCA, 2020). A epidemiologia é apoiada fundamentalmente por informações de morbimortalidade obtidas pelos Registros de Câncer de Base Populacional (RCBP), Registros Hospitalares de Câncer (RHC) e pelo Sistema de Informações sobre Mortalidade (SIM) do Departamento de Informática do Sistema Único de Saúde (DATASUS). A mais recente estimativa mundial, ano 2018, aponta que ocorreram no mundo 18 milhões de casos novos de câncer (17 milhões sem contar os casos de câncer de pele não melanoma) e 9,6 milhões de óbitos (9,5 milhões excluindo os cânceres de pele não melanoma). Nas mulheres, as maiores incidências foram câncer de mama (24,2%), cólon e reto (9,5%), pulmão (8,4%) e colo do útero (6,6%) (Bray *et al.,* 2018). Para o Brasil, a estimativa para cada ano do triênio 2020-2022 aponta que ocorrerão 625 mil casos novos de câncer, sendo 66.280 casos novos de câncer de mama, para cada ano do triênio 2020-2022. Esse valor corresponde a um risco estimado de 61,61 casos novos a cada 100 mil mulheres (INCA, 2020).

Dentre as abordagens terapêuticas para o câncer, encontra-se a radioterapia (RT) que utiliza a radiação ionizante, cujos efeitos imunossupressores causados pelas altas doses de radiação podem induzir à apoptose das células radio sensíveis, dentre as quais os linfócitos têm sido bem documentados (Falcão e cols, 2015; Brunt AM e cols 2016). Entretanto, doses fracionadas em um regime multifracionado de RT, as quais são utilizadas para tratamento de alguns tipos tumorais, podem regular positiva ou negativamente as funções de distintas células imunes através de mecanismos de regulação, tais como enzimas que ora desencadeiam a transcrição de novos genes tumorais, bem como são também capazes de inibir a transcrição dos mesmos

(Stankevicins L e cols, 2013). Sabe-se que a radioterapia apresenta grande eficiência sendo uma abordagem terapêutica capaz de destruir células tumorais, empregando feixe de radiações ionizantes. No entanto, estas radiações, podem interagir com os tecidos, dando origem a elétrons rápidos que ionizam o meio e criam efeitos químicos tais como a hidrólise da água e a ruptura das cadeias de DNA. As interações causadas por estas radiações podem resultar em morte celular através de vários mecanismos, que passam pela inativação de sistemas vitais para a célula até sua incapacidade reprodutiva (INCA, 2020).

Embora o padrão-ouro para o tratamento do câncer de mama possa ser considerado a cirurgia, muitas pacientes podem desejar evitá-la. No estudo de Tagliaferri e cols (2019) pacientes foram tratadas por irradiação cobrindo toda a mama de forma convencional seguida por radioterapia estereotáxica (tumor primário apenas) ou reforço de radioterapia de intensidade modulada, no tumor mais nódulos axilares. Apesar da comprovada eficácia dos tratamentos utilizados atualmente, casos de intolerância e insucesso são recorrentes. Isso porque a grande dificuldade no tratamento do câncer é o fato de suas células tumorais se adaptarem e sobreviverem a vários tipos de terapias, levando à resistência (Khan FM, 2010; Maria, 2013; Falcão *et al.*, 2015). Dentre às linhagens tumorais submetidas à radioterapia consideradas potencialmente mais agressivas, podendo produzir recidivas, vale mencionar a linhagem de adenocarcinoma de mama MDA-MB-231 radio resistente, que necessita altas doses de radiação para a redução de sua viabilidade celular (Falcão *et al.*, 2015).

Neste contexto, concomitantemente ao tratamento com a radioterapia, a paciente pode ser induzida ao um estado de imunossupressão, devido à morte das células mononucleares do sangue periférico (PBMCs), principalmente de linfócitos T auxiliadores $CD4^+$ durante o tratamento, sendo neste caso consideradas populações celulares radio sensíveis. Estas células desempenham papel importante na defesa do organismo durante o tratamento radioterápico, fornecendo aporte fisiológico ao paciente para resistir ao tratamento, reduzindo a própria imunossupressão e consequentemente potencializando a resposta imunológica da paciente durante e após o tratamento (Falcão *et a.l*, 2015).

A investigação de terapias adjuvantes ao tratamento de radioterapia tem aumentado, a fim de reduzir as complicações causadas por essa abordagem, sendo um deles é o uso de radioprotetores (Mun *et al*, 2018). No entanto, na prática clínica alguns fármacos têm uso limitado devido aos seus efeitos colaterais e alta toxicidade, como por exemplo, hipocalemia e reações anafiláticas. O desenvolvimento de radioprotetores eficazes e menos tóxicos é de grande interesse, o que tem estimulado o estudo com compostos de origem natural (Weiss & Landauer, 2009).Tung-Kwang Lee *et al.*, 2010)

Na expectativa de reduzir tais efeitos, muitas substâncias que ocorrem naturalmente na natureza têm sido consideradas como candidatas à radioproteção, e nesse grupo destacam-se os antioxidantes que são substâncias encontradas em muitos produtos naturais e possuem capacidade de neutralizar os efeitos causados pela radiação, dentre eles os que compõem a própolis. Dessa forma, a investigação da ação radioprotetora da própolis tem aumentado ao longo da última década, e seus efeitos têm sido extensivamente abordados em estudos *in vitro e in vivo* (Benkovic *et al*., 2009, Batista et al, 2018). Atualmente, a utilização de própolis como uma substância imunomoduladora tem sido considerada uma alternativa para a prevenção e cura de diversas enfermidades (Orsolic, 2003).

A ação imunomoduladora associada à utilização da própolis tem sido observada tanto na estimulação como na supressão de determinados eventos da resposta imune, tornando-a potencialmente aplicável como uma substância imuno estimulante, ou no combate a processos inflamatórios indesejáveis.

Embora existam vários estudos abordando a influência da própolis sobre o sistema imunológico, muitos resultados não são complementares ou mesmo podem ser considerados antagônicos, provavelmente em função de diferenças metodológicas ou da grande diversidade química entre as amostras de própolis utilizadas. Há de se mencionar que estudos mais recentes têm sugerido que as propriedades bioativas da própolis podem estar associadas inclusive às formas de extração da mesma. Vários métodos são usados em todo o mundo para extrair os componentes da própolis; entretanto, a extração usando etanol como solvente é o método mais usado (Cao et al, 2017). Extratos etanólicos têm sido mais comumente usados devido ao seu conteúdo em ácidos fenólicos e flavonóides (Cao et al, 2017). Outros métodos têm sido utilizados para

aumentar a eficiência da extração dos componentes bioativos da própolis, como a ultrassonografia e a extração assistida por microondas e a extração de fluido supercrítico (Cao et al, 2017; Catchpole et al, 2004; Pellati et al, 2013). Vale mencionar que o método tradicional de extração com etanol foi adotado nos procedimentos propostos neste trabalho.

1.2 Objetivos

1.2.1 Objetivo geral

O objetivo geral foi avaliar o papel do extrato da própolis de T*etragona clavipe*s e seu mecanismo de ação sobre a viabilidade celular e mediadores pró e anti tumorais em células de adenocarcinoma de mama MDA-MB 231 radio resistente e células mononucleares do sangue periférico (PBMC).

1.2.2 Objetivos específicos

- Avaliações dos efeitos do extrato da própolis sobre a viabilidade de células da linhagem de adenocarcinoma de mama MDAMB-231 e células mononucleares do sangue periférico humano (PBMCs) *in vitro* em cinética de doses de Cobalto-60;

- Avaliação do efeito de mediadores pro e anti-tumorais sobre células de adenocarcinoma de mama MDA-MB-231 radio resistente.

1.3 Motivação e Ineditismo

A Própolis é conhecida por conter uma variedade de compostos químicos como esteróides ácidos fenólicos, ésteres de ácidos fenólicos, flavonóides e terpenóides, como CAPE e Artepillin C (Huang et al 2014). Adjuvantes fitoterápicos envolvendo o uso da própolis têm sido descritos na literatura e têm mostrado sua eficiência sobre a

clonogênese de tumores e como radioprotetor não tóxico. O principal componente químico presente na própolis é o éster fenetílico ácido cafeíco (CAPE). Foi comprovado que este composto possui atividades biológicas importantes, incluindo antibacteriana, antiviral, antioxidante, anti-inflamatória e anti-tumoral (Park et al 2009).

A própolis tem demonstrado eficácia contra câncer de cérebro, cabeça e pescoço, pele, mama, fígado, pâncreas, rim, bexiga, próstata, cólon e sangue. A inibição das metaloproteinases da matriz, antiangiogênese, prevenção da metástase, parada do ciclo celular, indução de apoptose e moderação dos efeitos colaterais deletérios induzidos pela quimioterapia foram deduzidas como os principais mecanismos de manipulação do câncer (Pattel S, 2016). Os componentes da própolis já descritos têm demonstrado uma eficiência antitumoral, incluindo também a crisina, artepilina C, nemorosona, galangina, cardanol etc. Esses compostos têm como alvo várias vias genéticas e bioquímicas da progressão do câncer. Dependendo das fontes botânicas e da origem geográfica, as atividades biológicas da própolis variam. Apesar do desenvolvimento fenomenal na pesquisa do câncer, a terapia convencional é insuficiente no tratamento completo da malignidade. Os resultados obtidos até agora aumentam a esperança de que a própolis como medicamento complementar possa sanar as lacunas no tratamento do câncer (Pattel S, 2016).

Dessa forma, a suplementação de própolis com tratamento de radioterapia parece oferecer uma proteção bastante mensurável contra danos ao DNA causados pelas radiações ionizantes em leucócitos de pacientes durante o tratamento de radioterapia (Ebeid et al 2016). Ademais, a atividade imunológica proporcionada pelo uso da própolis e compostos relacionados aumenta a regeneração hematopoiética e sobrevivência após indução por radiação (Orsolic et al 2007). Vale mencionar outros estudos que forneceram suporte para direcionar novas abordagens à própolis em ensaios *in vitro,* a exemplo de pesquisadores que investigaram a atividade anti-HIV significativa da própolis em cultura de linfócitos H9, enquanto, outros estudos observaram uma atividade anti-clonogênica do extrato etanólico de própolis, no crescimento de células do tumor primário, bem como do metastático da próstata, bem como descreveram atividade antiviral de influenza pela própolis da região sul (AF-08) em modelo animal (Ito et al. 2001; Shimizu *et al* 2008). O estudo de Shimizu e cols (2019) sugeriu que o extrato de própolis brasileira reduz defeitos de barreira intestinal e inflamação em um

modelo de camundongo com colite e demonstrou um papel na regulação da barreira de junções estreitas (TJ), diferenciação de células Th17 e / ou ativação de macrófagos por derivados do ácido cinâmico estão envolvidos no efeito anti-inflamatório mediado pela própolis.

Yan *et al,* 2020, fizeram um estudo comparativo entre a própolis chinesa e a própolis verde brasileira em relação ao perfil metabólico e bioatividade. A ChPs (Própolis Chinesa) e BrGPs (Própolis Verde Brasileira) mostraram potencial anti-inflamatório semelhante. Curiosamente, os resultados mostraram que eles continham níveis muito diferentes de extrato de etanol, flavonoides totais, e ácidos fenólicos totais. De fato, o perfil metabólico em LC-MS pode efetivamente distinguir a ChPs e a BrGPs. (Yan *et al,* 2020). A literatura mostrou recentemente ainda a influência dos métodos de extração da própolis assistida por ultrassom, farmacopeia e fluido supercrítico sobre os compostos bioativos e as atividades biológicas da própolis. Os resultados mostraram que a própolis extraída pelo método assistido por ultrassom continha mais compostos fenólicos, e apresentou o maior conteúdo fenólico total, conteúdo total de flavonoides e mais forte em atividade antioxidante *in vitro* do que aqueles de métodos de farmacopeia e fluido supercrítico. (Yuan *et al,* 2019)

A duas décadas atrás, havia já sido reportado que a composição assim como a coloração da própolis depende da flora da região visitada pela abelha (Park et al., 2002). E mais recentemente em 2014, Bankova e colaboradores mostraram numa revisão a grande variabilidade na sua composição (Bankova et al., 2014; Visweswara Rao Pasupuleti *et al,* 2017). Diante disso, a caracterização qualitativa e quantitativa dos componentes da própolis tem subsidiado a investigação da eficácia biológica da própolis de diferentes procedências e seus efeitos imunomoduladores

(Nogueira, dissertação de mestrado, 2017). A despeito do amplo espectro de atividades da própolis e da sua reconhecida importância à saúde humana, as informações na literatura sobre a própolis, especificamente brasileira, proveniente da região norte do Brasil ainda são perenes (Santos, 2011).

Por tanto, se tornam promissores os estudos acerca da utilização da própolis e seus efeitos sobre os danos decorrentes da radiação gama (γ) sobre células de adenocarcinoma de mama MDA- MB- 231 radio resistente e seu mecanismo molecular,

bem como seu papel sobre a produção de mediadores pró e antitumorais. Vale também a pena reportar que estudos sugeriram um papel protetor do extrato da própolis sobre células do sistema imunológico (SI) em culturas *in vitro*, e dentro do contexto de que as células do SI que permanecem adjacentes ao tumor podem desempenhar um papel crucial na contenção dos efeitos mórbidos do tumor, sendo fundamental a manutenção da viabilidade destas células do SI. (Nogueira, Tese de mestrado, 2017).

Dentro do contexto deste estudo, espera-se esta contribuição possa trazer uma melhor compreensão do papel da própolis no tratamento coadjuvante de tumores, bem como fornece também um suporte para novos planejamentos radioterápicos futuros de pacientes sob tratamento.

1.4 Organização do documento de Qualificação e Tese

Este é o documento apresentado para defesa de tese de doutorado, o qual representa o conjunto de trabalhos desenvolvidos durante o período de doutoramento pelo programa de Pós-graduação em Ciências e Técnicas Nucleares (PCTN), dando ênfase ao estudo do papel da própolis no tratamento do câncer de mama, em estudos *in vitro*, sendo sua organização apresentada sob a forma de artigos científicos.

Neste primeiro capítulo, foi apresentado o tema, os objetivos e o ineditismo da proposta. No capítulo 2, seguinte, descrevemos o estado da arte, dando ênfase à descrição da fisiopatologia do câncer de mama, os achados sobre a radioterapia, radioprotetores e finalmente a descrição dos flavonoides e da própolis. No capítulo 3 descrevemos a metodologia realizada para os dois artigos experimentais descritos nos capítulos 4 e 5, respectivamente. O capítulo 4 corresponde ao artigo preparado com os resultados do estudo do papel do extrato de própolis *Tetragona clavipes* na inibição de interleucinas pró-tumorais produzidas pela linhagem de adenocarcinoma de mama MDAMB231 irradiadas com Co-60 de baixa dose. Já o capítulo 5 corresponde ao artigo gerado para a caracterização bioquímica do extrato aquoso da própolis da espécie *Tetragona clavipes* e sua utilização nos experimentos *in vitro*, em colaboração com Universidade Federal do Amazonas (UFAM). Neste artigo, os experimentos *in vitro referente a*os resultados produzidos foram realizados diretamente no Laboratório de

Radiobiologia – Núcleo de Radiações Ionizantes – NRI, dentro do programa PCTN, sob a supervisão da proponente.

Os capítulos 6 e 7 correspondem a duas revisões, que inclusive forneceram suporte para discussão dos dados experimentais. A primeira revisão (Capítulo 6) discorre sobre o papel de interleucinas, em particular, as citocinas envolvidas na modulação de respostas imunes no tumor de mama e doenças idiotípicas, incluindo a interleucina 10 (IL-10). O capítulo 7 apresenta um "short *review"* sobre as terapias atuais do câncer e sua associação, considerando sua grande relevância para o contexto do tema principal da tese. Finalmente, no capítulo 8 apresentamos a conclusão geral de uma forma holística, vista que nesta foram pontuados o conjunto dos achados mais relevantes obtidos nos estudos experimentais, bem como as perspectivas para a continuidade do estudo no futuro.

CAPÍTULO 2

ESTADO DA ARTE

2.1 Câncer de mama

Como nos reportamos na descrição do tema da tese, no Brasil estimam-se que 66.280 casos novos de câncer de mama, para cada ano do triênio 2020-2022. Esse valor corresponde a um risco estimado de 61,61 casos novos a cada 100 mil mulheres. O câncer ocorre através do resultado de falhas cumulativas em um dos mais organizados sistemas do organismo que é de controle multiplicativo de células. As células cancerosas apresentam característica instável conferindo malignidade às mesmas, influenciando primariamente o poder de invasão e disseminação que o tumor possui, podendo atingir limites adjacentes, sistema linfático e a corrente sanguínea (Bray, 2018).

O número de mulheres que morrem por câncer de mama tem aumentado, e em nível mundial e esta doença perde o lugar apenas para o câncer de pulmão (INCA - 2020). Segundo os dados levantados pelo Instituto Nacional de Câncer – INCA (2020), os principais sinais e sintomas de câncer de mama são nódulos na mama e/ou axila, dor mamária e alterações da pele que recobre a mama, como abaulamentos ou retrações com aspecto semelhante à casca de laranja.

De acordo com Smeltzer *et al.* (2006) os tumores de mama localizam-se, principalmente, no quadrante superior externo, e em geral, as lesões são indolores, fixas

e com bordas irregulares, acompanhadas de alterações da pele quando em estágio avançado.

Essa doença pode ser controlada através da detecção precoce, na qual a lesão se restringe ao parênquima mamário, com um tamanho de no máximo três centímetros, permitindo o uso de recursos terapêuticos menos mutiladores e maior possibilidade de cura. (INCA, 2020). Dentre os exames realizados para a detecção precoce de câncer de mama estão: o exame clínico de mamas (ECM), mamografia e o autoexame de mamas (AEM) (INCA, 2020).

Embora o avanço na medicina tenha sido significativo nas últimas décadas, o diagnóstico precoce, a terapia adequada e os resultados favoráveis do tratamento do câncer continuam sendo muito desafiadores. Portanto, o câncer ainda representa uma das principais preocupações de saúde pública, pois é uma doença complexa, heterogênea e muito agressiva (Kaufmann J.K. *et al*, 2014; Silva C.O. *et al*, 2019). Segundo a American Cancer Society, o câncer é responsável por cerca de uma em cada seis mortes no mundo. Espera-se que o número de novos pacientes com câncer em 2040 seja de 27,5 milhões (Kaufmann J.K. *et al*, 2014). Em outras palavras, isso significaria quase o dobro dos 14,1 milhões em 2012. Os dados para os EUA sobre uma taxa de sobrevida relativa de 5 anos para todos os cânceres combinados mostram resultados muito melhores desde o início dos anos 1960. A porcentagem aumentou de 39% para 70% na população caucasiana e de 27% para 63% nos afro-americanos. Embora os números pareçam muito promissores e provavelmente reflitam uma melhor compreensão da biologia do tumor, avanços no tratamento e diagnóstico precoce de alguns tipos de câncer, o próprio curso do tratamento, bem como o longo acompanhamento de possíveis recidivas ainda são fatores cruciais que influenciam drasticamente sobre a taxa de sobrevida (Kaufmann J.K. *et al*, 2014; Peer J. *et al*, 2007; Quader S. *et al*, 2017).

A base do tratamento para o câncer é composta essencialmente pela intervenção cirúrgica, radioterapia, quimioterapia, e atualmente, vem se desenvolvendo propostas de uso de nanoterapias (Cunha *et al*, 2010).

A escolha da terapia para o câncer pode ocorrer com base na utilização de um método apenas, ou promovendo a combinação entre os métodos, visando à destruição das células cancerosas, porém, tanto a radioterapia como a quimioterapia, atingem células normais, fazendo com que o tratamento, muitas vezes, ocorra em intervalos regulares que possibilitem a recuperação das células normais do tecido acometido (Vanneman, M & Dranoff, G., 2012).

2.2 Tratamentos e/ou terapias associadas

Radioterapia (RT) é a modalidade mais comum para o tratamento de câncer humano, cerca de 80% dos pacientes com câncer precisam ser submetidos à radioterapia em algum momento ou outro, seja para fins de curativo ou paliativo (Paul *et al.*, 2011).

A radioterapia usa radiações gama (γ) no tratamento do câncer. A radiação ionizante causa danos diretos ao DNA das células do corpo humano, com o rompimento parcial ou total da dupla hélice. O objetivo da radioterapia é induzir a morte de células tumorais com uma alta dose, danificando o mínimo possível os tecidos saudáveis vizinhos (Murad; Katz, 1996).

Os efeitos das radiações em mamíferos vêm sendo estudados nos últimos 20 anos, através da análise de diferentes mecanismos como danos e reparos ao ácido desoxirribonucléico (DNA), reações antioxidantes, apoptose e danos celulares, mutagênese e respostas imune adaptativa (Liu *et al.*, 2003). O tratamento com radiação em longo prazo provoca efeitos tanto ao sistema imune adaptativo quanto ao inato (Liu, 2003).

Conforme Standish *et al.* (2008), em seu estudo realizado para avaliar o estado imune de mulheres que se submeteram ao tratamento de câncer de mama com RT, mostrou que as pacientes apresentaram déficits imunológicos, como a redução da secreção de imunoglobulinas IgM, IgA e IgG; outras pacientes apresentaram linfopenia; diminuição no número total de células T; baixa atividade natural das células assassinas (NK) e baixos níveis de citocinas apoptóticas foram associados em alguns casos após RT.

A exposição à radiação ionizante causa quebra de fita simples do DNA, rutura de filamentos duplos, causando dano nas bases de DNA-proteína e ligações cruzadas no DNA genômico. Entre eles, a rutura de filamentos duplos é a lesão mais crítica, que pode levar a instabilidade genómica e morte celular. Os diversos efeitos em células e tecidos, especialmente no DNA são bastante conhecidos através de estudos sobre células isoladas, tecidos e todo organismo após a exposição em altas doses (Scott, 2003; Standish, 2008).

O Cobalto caracteriza-se como uma radiação de baixo LET (*Linear Energy Transfer*) **e** vem sendo comumente utilizado em tratamento de RT. Enquanto radiação ionizante mesmo em baixas doses e com baixas taxas de doses, seus efeitos no sistema imune podem ser supressores ou estimulatórios (UNSCEAR, 2008).

Esses efeitos em duas vias podem ser esclarecidos através da produção de espécies reativas de oxigênio ROS e seus diversos tipos que potencialmente podem mudar a sinalização intracelular e expressão gênica. As diferentes categorias de adaptações celulares a agentes tóxicos e de proteção correspondente, como desintoxicação ROS, reparo no DNA, incluindo a apoptose pode ser estimulada por ROS, porque eles ocorrem em diferentes taxas ao longo do organismo (Bauer, 2000).

Acredita-se que o aumento da resposta imune inata e específica é um dos mecanismos da eficácia da radiação de corpo inteiro com baixas doses (Safwat, 2000; Safwat, 2001). As consequências do aumento da resposta imune inata são a ativação da resposta inflamatória caracterizada por infiltrados de neutrófilos e mudanças na ultraestrutura característica de macrófagos no tecido alvo, a ativação desses fagócitos caracterizada por aumento da atividade lisossomal e da enzima Óxido Nítrico Sintase (NOS) e de ROS caracterizando como uma resposta específica (Lorimore *et al.*, 2001).

Sabe-se que os danos celulares são atribuídos primariamente aos efeitos maléficos dos radicais livres. Sendo assim, perdura há mais de seis décadas a busca por agentes protetores ideais que desempenhem o papel de diminuir esses danos a exposição à radiação. Em relação aos radio protetores, podemos mencionar que esses compostos possuem propriedades de neutralizar estes radicais são considerados particularmente promissores como agentes radioprotetores (Santos, 2011).

Essas substâncias agem através da via “scavenging” de radicais livres produzidos pela radiação. Levando-se em consideração a vida extremamente curta dos radicais livres, estes agentes necessitariam estarem presentes no meio celular, preferencialmente, antes da exposição à radiação para neutralizar as propriedades destrutivas dos radicais livres (Hosseinimehr, 2007). Os radio protetores mais conhecidos são os compostos aminotióis, como cisteína, cisteamina e AET (brometo de 2-aminoetilisotiuréia) (Hall, 2000; Weiss e Landauer, 2009).

Dessa forma, há necessidade de identificar compostos não tóxicos, efetivos e convenientes para proteção humana dos efeitos adversos da radiação ionizante. Diversos compostos têm sido testados para a sua eficácia radioprotetora. Todavia, todos esses compostos produzem efeitos colaterais indesejáveis, como náusea e vômitos e são considerados tóxicos nas doses necessárias para a radioproteção, o que limita o seu uso na prática médica (Vijayalaxmi *et al*, 2004; Shirazi *et al*, 2007).

Atualmente, muitos compostos sintéticos testados apresentam um sucesso limitado, substâncias com propriedades antioxidantes que ocorrem naturalmente, como enzimas endógenas (glutationa, superóxido dismutase, catalase) ou hormônios (melatonina), vitaminas (vitaminas C e E), carotenoides e flavonoides, têm despertado um interesse particular cada vez maior (Weiss & Landauer, 2009).

Os flavonoides têm sido alvo de grande interesse científico e terapêutico, devido as suas propriedades. Devido a uma modulação da função imunológica os mesmos têm sido sugeridos como envolvidos no papel desempenhado por alimentos vegetais na prevenção de doenças. Estudos realizados para avaliar o efeito de alimentos ricos em flavonoides e suplementos de flavonoides na função imunológica tem investigado seu papel sobre citocinas pró-inflamatórias e marcadores *ex vivo* da função imunológica (Peluso *et al.,* 2015). No entanto, de acordo com os últimos achados e com base em itens alimentares individuais, o número de estudos em humanos é limitado e, para suplementos galênicos, apenas a quercetina como fonte de investigação. Mais evidências são necessárias para esclarecer o papel dos flavonóides como moduladores da função imunológica em humanos (Peluso *et al.,* 2015). Os resultados obtidos através de estudos epidemiológicos têm mostrado uma associação direta entre o consumo de frutas/

vegetais e o risco diminuído de vários tipos de câncer, incluindo o de mama, cólon, laringe, pâncreas e próstata (Ross & Kasum, 2002; Rithidech *et al.*, 2005). Esses possíveis efeitos protetores de flavonoides, juntamente com suas atividades antioxidativas e "scavenger" de radicais livres, observados tanto em estudos *in vivo* (Orsolic *et al.*, 2007; Benkovik *et al.*, 2008a) como *in vitro* (Benkovik *et al.*, 2008b), tem propiciado um interesse público sobre o consumo de flavonoides para seus potenciais benefícios à saúde.

A concentração de flavonoides nos vegetais de modo geral é baixa e a sua extração é extremamente trabalhosa, o uso de própolis tem sido uma alternativa atrativa, pois este produto das abelhas contém uma concentração mais alta de flavonoides, cerca de 25 – 30 % do seu peso seco (Krol *et al.*, 1990; Benkovik *et al*, 2008b).

A própolis é um produto resinoso encontrado na colméia (Figura 1), coletado por abelhas de várias fontes de planta e é utilizada pelas abelhas para selar eventuais aberturas na colméia e para eliminar possíveis invasores (Banskota, 2001).

Figura 2.1- (A) Própolis depositada no interior da colmeia; (B) deposição de própolis na tampa da colmeia da abelha Borá (*Tetragona clavipes)* **Fonte:** (Nogueira, 2017)

Segundo Havsteen (2002), em uma amostra típica de própolis são encontrados cerca de 25 diferentes tipos de flavonoides em concentrações significativas, o que sugere que o extrato de própolis retém a maioria das propriedades bioquímicas comumente associadas aos flavonoides, isto é, efeito antioxidante e "scavenging" de radicais.

Neste sentido, tem aumento o interesse no cultivo de abelhas, devido seus produtos, dentre os produtos apícolas, a própolis, que vem sendo usada em medicina popular por séculos, vem se destacando tanto pelas suas propriedades terapêuticas como atividades antimicrobiana (Daugsch *et al.*, 2007), anti-inflamatória, antioxidante, imunoestimuladora, cicatrizante, anestésica e anticarcinogênica (Park *et al.*, 1998), quanto pela possibilidade de aplicação nas indústrias farmacêuticas e alimentícias. Essas propriedades farmacológicas estão relacionadas a mais de 300 compostos que têm sido identificados em própolis (Ishihara *et al.*, 2009).

A investigação realizada na própolis originária da Europa e China possibilitou a identificação de vários tipos de flavonoides e ésteres de ácido fenólico (CAPE) (Figura 2), enquanto a própolis brasileira é composta principalmente de artepilina C (ácido 3,5-diprenil-4-hidroxicinâmico), terpenóides e ácido p-cumárico (Chen *et al.*, 2004).

Artepilina C (ácido 3,5-diprenil-4-hidroxicinâmico) é o principal componente biologicamente ativo fenólico encontrado na própolis verde, coletado da planta *Baccharis dracunculiforia* no Sudeste do Brasil (Park *et al*., 2004; De Souza *et al.*, 2011). Artepillin C possui ação antioxidante, antimicrobiana, antiinflamatória, antigenotóxica, antiangiogênica e propriedades anticancerígenas (Matsuno *et al*., 1997; Watanabe *et al*., 2011. Como mencionado anteriormente, os diferentes tipos, a própolis chinesa (ChPs) e a própolis verde brasileira (BrGPs) demonstraram conter propriedades multifuncionais. (De Souza *et al.* 2019).

A própolis procedente da região sul do Brasil não contém artepelina C na sua composição, mas é relativamente rica em flavonóides, incluindo galangina, apigenina, crisina, pinobanksina, pinobanksina-3 e pinocembrina (Liu *et al.*, 2007). A principal origem botânica da própolis dessa região foi identificada como sendo *Myrceugenia eurosma* (Myrtaceae), um cultivo abundante no sul do Brasil, quase na fronteira com o Uruguai. (Ito *et al.*, 2001).

2.3 Células de linhagem tumoral – adenocarcinoma de mama MDA-MB-231

A primeira linhagem celular humana foi utilizada em um laboratório de Baltimore há mais de 50 anos por George Gey (Shooter & Gey GO *et al*., 1952). Esta linhagem celular recebeu o nome de *HeLa* - após ser doada por Henrietta Lacks, a senhora de quem derivava a linha celular, que tinha carcinoma cervical. A visão de Gey abriu o caminho para a cultura de células como a conhecemos hoje, permitindo que o seu desenvolvimento generalizado seja uma importante ferramenta experimental na pesquisa do câncer. Um dos principais benefícios do uso de linhagens de células cultivadas na pesquisa de câncer é que eles oferecem um suprimento infinito de uma população de células relativamente homogêneas que é capaz de autorreplicação em meio de cultura celular padrão (Holliday & Speirs, 2011).

Em relação ao câncer de mama, a primeira linhagem celular a ser estabelecida foi BT-20 em 1958 (c). No entanto, foram mais 20 anos, antes de estabelecer linhagens de câncer de mama, mais difundidas, incluindo a série MD Anderson (Cailleau *et al.*, 1978). Estas células foram isoladas por Cailleau e colegas em 1973, a partir de uma amostra de efusão pleural de pacientes com câncer da mama que morreram desta doença no Hospital Anderson (Houston, EUA) (Cailleau et al 1974).

Muito antes do surgimento das modernas técnicas de perfil molecular, os histopatologistas reconheceram que o câncer de mama era heterogêneo através de observações morfológicas. A classificação baseou-se nas seguintes medidas: tipo histológico, grau tumoral, estado dos linfonodos e presença de marcadores preditivos, como ER e, mais recentemente, receptor do fator de crescimento epidérmico humano 2 (HER2). O desenvolvimento de perfis moleculares usando microarrays de DNA provou essa heterogeneidade, demonstrando através do perfil de expressão gênica e a expressão imuno-histoquímica de ERα, receptor de progesterona (PR) e HER2 que o câncer de mama pode ser classificado em pelo menos cinco subtipos: luminal A, luminal B, HER2 , Basal e normal (Perou *et al*. 2000).

Neste contexto, a linhagem de adenocarcinoma de mama MDA-MB-231 (Figura 2) é classificada como um subtipo basal, os tumores basais são difíceis de tratar, são mais biologicamente agressivos e muitas vezes têm um mau prognóstico, esta linhagem celular têm apresentado resistência a quimioterapia e radioterapia (Holliday & Speirs, 2011; Falcão et al. 2015). Como o fenótipo basal é caracterizado pela falta de expressão de ERα, PR e HER2, em alguns casos é referido como triplo negativo (Holliday & Speirs, 2011).

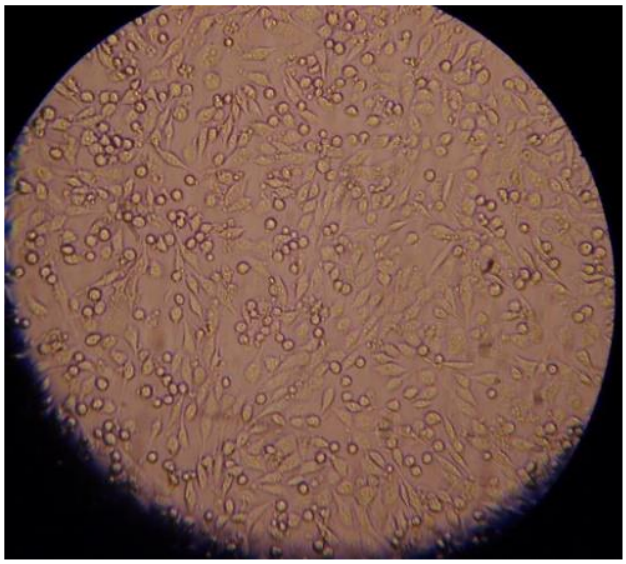

Figura 2.2 – Microscopia óptica células de MDA-MB-231

Fonte: Falcão *et al*. 2015

A linhagem de adenocarcinoma de mama MDA-MB-231 é a mais utilizada para o estudo experimental *in vitro* de câncer da mama hormono-independente. Verificou-se que estas células têm um rápido crescimento em meio de cultura enriquecido, em parte, por uma regulação autócrina de fatores de crescimento de células que se segregam para o meio. Eles possuem receptores de membrana abundantes para o fator de crescimento epidérmico (EGF) (Martínez-Carpio *et al*., 1999; Belkaid & Harrison, 2017). Estudos bioquímicos e genéticos com estas células têm contribuído grandemente para a pesquisa do câncer da mama e o desenvolvimento de drogas para ajudar a combatê-lo (Holliday & Speirs, 2011).

2.4 Peripheral Blood Mononuclear Cells (PBMC) - Células Mononucleares do Sangue Periférico

O Sistema Imunológico (SI), assim como os outros sistemas orgânicos, tem função fisiológica, atuando no sentido de manter a homeostasia do organismo, alterada por estímulos induzidos por fatores intrínsecos ou extrínsecos Falcão *et al*, 2015. A resposta imune contra fatores externos, incluindo patógenos, e fatores internos, como células tumorais, tem sido estudada ao longo dos tempos como sendo responsável pelos mecanismos de defesa. Para isto o SI conta com células e moléculas que atuam de maneira inespecífica, denominada imunidade inata (II), atuando como primeira linha de defesa (entenda-se: retorno à homeostasia) contra patógenos, enquanto a permanência destes é capaz de estimular outras células -linfócitos- que se adaptam a estes estímulos externos, tornando-se mais específicas na tentativa de dar continuidade a homeostasia do Organismo. (Vesely MD *et al.* 2011).

A microbiota desempenha um papel fundamental na indução e função do sistema imunológico do hospedeiro. Em troca, o sistema imunológico do hospedeiro desenvolveu vários meios pelos quais mantém sua relação simbiótica com a microbiota. A manutenção desse diálogo permite a indução de respostas protetoras a patógenos e a utilização de vias regulatórias envolvidas na tolerância sustentada a antígenos inócuos. A capacidade dos microrganismos de definir o papel imunológico dos tecidos, tanto local quanto sistemicamente, requer a detecção dos microrganismos e circuitos de feedback complexos entre os componentes inatos e adaptativos do sistema imunológico.(Belkaid Yasmine & Harrison Oliver J, 2017)

Por outro lado, vale a pena mencionar que metabólitos sintetizados pelas células do sistema imune tem papéis importantes na indução das respostas imunes inata e adaptativa, como é o caso da vitamina D, cujo receptor é expresso nas células imunes (células B, células T e células apresentadoras de antígeno), e essas células imunológicas são todas capazes de sintetizar o metabólito ativo da vitamina D, que tem a capacidade de agir de maneira autócrina em um meio imunológico local, modulando as respostas imunes inatas e adaptativas. A deficiência de vitamina D está associada ao aumento da autoimunidade e ao aumento da suscetibilidade à infecção. (Aranow, 2011)

As células mononucleares do sangue periférico (PBMC) são populações de células sanguíneas do sistema imune que possuem um núcleo redondo. Essas células consistem emlinfócitos (Células T, Células B e Células NK (Natural Killer) e macrófagos (Grievink *et al.* 2016) As técnicas de rotina para o isolamento de células mononucleares de sangue periférico humano (PBMCs) incluem centrifugação de densidade com Ficoll-Paque e isolamento por tubos de preparação de células (CPTs) e tubos SepMate com Lymphoprep. Em uma série de experimentos, essas três técnicas de isolamento de PBMC foram comparadas quanto à recuperação e viabilidade celular, composição da população de PBMC e funcionalidade celular, com o objetivo de fornecer uma base inicial para a seleção do método mais apropriado de isolamento de PBMC para uma aplicação específica. PBMCs foram isolados recentemente de sangue venoso de doadores saudáveis do sexo masculino, aplicando as diferentes técnicas em paralelo. A recuperação e a viabilidade celular foi avaliada usando um hemacitômetro e azul de tripano. A imunofenotipagem foi realizada por citometria de fluxo. (Grievink *et al.* 2016) A funcionalidade celular foi avaliada em culturas de PBMC estimuladas (100 ng / mL de enterotoxina B estafilocócica [SEB] e não estimuladas de 24 horas, com produção de citocinas e liberação de lactato desidrogenase (LDH) como medidas de leitura. O isolamento de PBMC por SepMate e CPT resultou em uma recuperação 70% maior do que o isolamento de Ficoll. As populações isoladas de CPT continham mais contaminação de eritrócitos. A viabilidade celular, avaliada por exclusão de azul de tripano, foi de 100% para todas as três técnicas de isolamento. O isolamento de SepMate e CPT deu maiores respostas de citocinas induzidas por SEB em culturas de células, para IFNγ e para citocinas secundárias. A liberação de IL-6 e IL-8 em culturas não estimuladas foi maior para PBMCs isolados com CPT em comparação com PBMCs isolados com Ficoll e SepMate. A liberação de LDH não diferiu entre as técnicas de isolamento de células. Além de critérios como custo e praticidade de aplicação, esses dados podem apoiar a seleção de uma técnica de isolamento de PBMC específica para análise a jusante. Essa população de células tem sido largamente utilizada por muitos cientistas que realizam pesquisas nos campos da imunologia (incluindo distúrbios auto-imunes), doenças infecciosas , doenças malignas hematológicas , desenvolvimento de vacina s , imunologia de transplantes e triagem de alto rendimento. Em muitos casos, as PBMCs são derivadas de bancos de sangue (Grievink *et al.* 2016)

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CAPITULO 3

Metodologia

O capítulo 3 compreende a metodologia empregada nos experimentos correpondentes aos capítulos 4 e 5, que são os artigos em apresentamos os experimentos com culturas de células *in vitro,* com as linhagens de adenocarcinoma de mama e células mononucleares do sangue periférico (PBMC). Ainda estão sendo descritos no capítulo 3 a metodologia de pereparação do Extrato Aquoso da Própolis (EAP), bem como a descrição dos testes de Viabilidade Celular pelo MTT e os ensaios imunoenzimáticos (ELISA).

3.1- Preparação do Extrato Aquoso de Própolis (EAP).

A Própolis Crua foi obtida na Fazenda Poranga, Município de Itacoatiara, Estado do Amazonas, Brasil, da colmeia da espécie Tetragona clavipes. A amostra de Própolis foi mantida resfriada a -4 ° C. Posteriormente, foi preparado o extrato de Própolis, conforme descrito por Matsushige et al., 1996, com modificações. Em um Becker, foram adicionados 500mL de água purificada e 100g de Própolis crua triturada no liquidificador e mantida por 24h. A mistura foi aquecida a um banho de água a temperatura constante a 80 ° C durante 2h. Posteriormente, essa solução foi misturada no liquidificador, filtrada (filtro de gaze), e acoplada a um funil e um kitasato. O filtrado foi seco em banho-maria durante 48h. Por fim, o pó resultante foi ressuspendido em meio RPMI nas concentrações experimentais (0,5%, 1,0%, 5% e 10%).

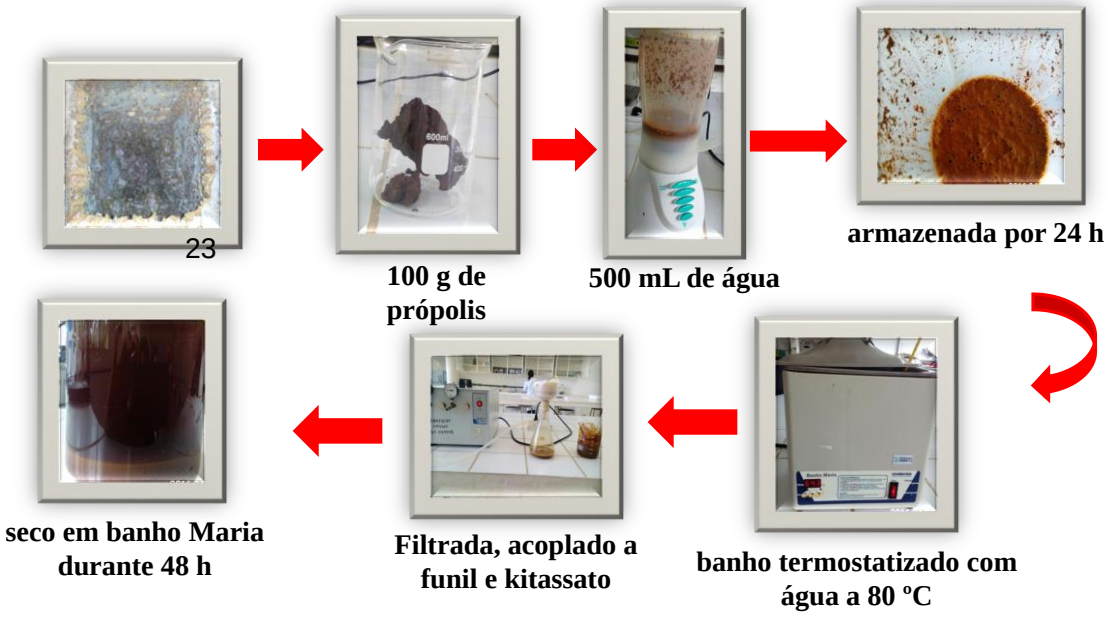


Figura 3.1- Sequência de procedimentos para extração e obtenção do Extrato Aquoso da Propolis (EAP).

3.2- Linhas pré-estabelecidas e manutenção da cultura *in vitro*

As Celulas Monucleares do sangue Perifèrico PBMC) foram obtidos de voluntários saudáveis foram colocados na cultura. As culturas celulares foram mantidas em meio RPMI-1640 suplementado com 10% de soro fetal bovino e antibiótico gentamicina (50µg.µL-1) e estreptomicina (500mg.mL-1), em frascos de cultura T-25 em atmosfera úmida contendo 5% de CO2 em 37 ° C.

A Separação de PBMC. As PBMCs dos pacientes foram separadas conforme procedimento descrito por Falcão *et al*., 2015. O sangue heparinizado foi aplicado em tubos de 15mL, siliconizados, contendo uma mistura de Ficolldiatrozato (Organon Teknika Corporation; Durham, NC), na proporção de um parte de Ficoll-diatrozato em duas partes de sangue. A solução foi submetida a centrifugação por 30min, em 1.400rpm em temperatura ambiente. No final do ciclo de spin, um anel de células mononucleares na interface entre o Ficoll e o plasma foi observado. Foi cuidadosamente removido com o auxílio de uma pipeta Pasteur e transferido para tubos de 15mL estéreis e lacrados (Falcon nº 2070). O volume de 15mL foi completado com solução de RPMI-1640 (Gibco®) não suplementado e submetido a nova centrifugação (10min, 1.200rpm). As células foram lavadas duas vezes (10 min, 1.200 rpm). Por fim, uma alíquota da

suspensão de células foi coletada e diluída (1:20) em tubo Eppendorf contendo 90µL de solução de Turck e o número de células determinado por contagem de câmaras de Neubauer, em microscópio óptico. A concentração de células foi ajustada para uma suspensão contendo 1,0 × 106 células.mL-1 com RPMI-1640 suplementado. Toda a manipulação das células foi realizada em condições estéreis, em cabina de fluxo laminar (Biological Cabinet BBL 60474 modelo).

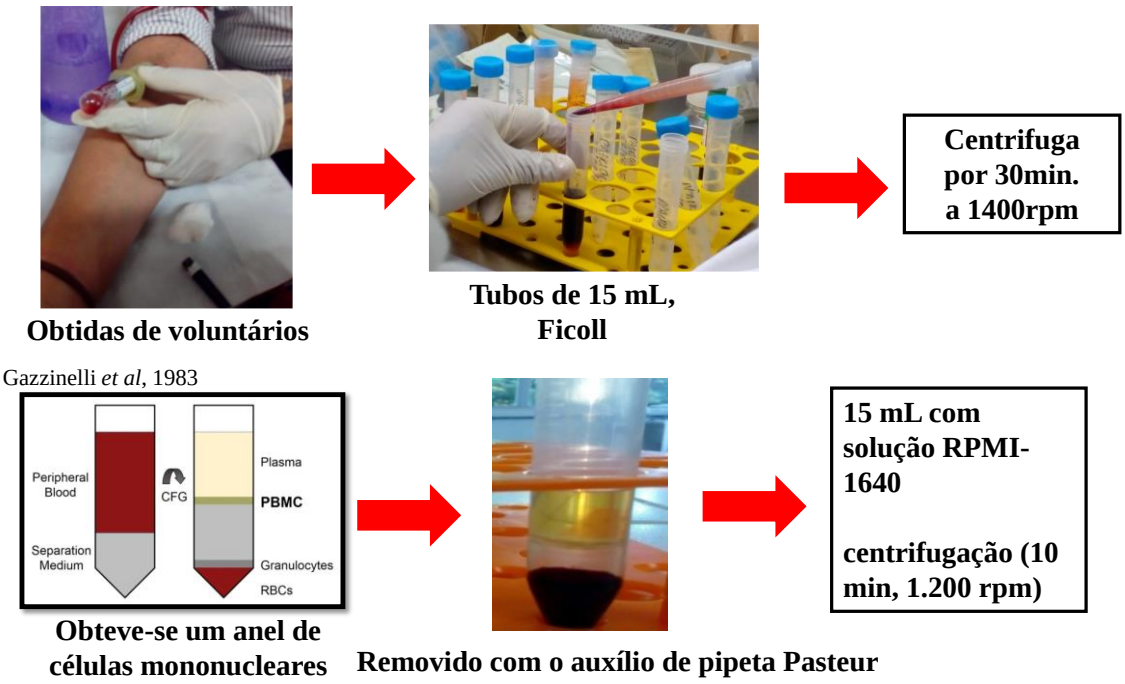


Figura 3.2- Sequencia de procedimentos para obtenção das Células Monucleares do Sangue Periférico (PBMC).

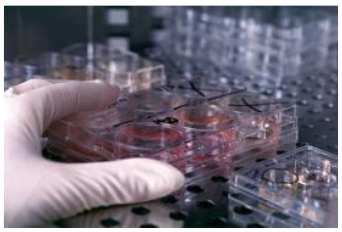
Controles de grupo. Os dois grupos controle foram preparados com PBMCs na condição de presença de extrato de própolis não irradiado e não aquoso, mantido em cultura conforme descrito anteriormente.

Cinética da dose de radiação. Frascos T25 contendo PBMC foram lacrados com parafilme e transportados em frasco plástico rígido com tampa, previamente descontaminado com álcool 70%, até o local da irradiação. Uma placa de tecido

equivalente de 4 cm foi colocada embaixo do frasco T25, bem como o meio de cultura foi preenchido com até 5 mm de altura, em relação à superfície das células aderidas. Esse procedimento foi necessário para atingir o equilíbrio eletrônico e garantir as doses prescritas nas células aderentes. Frascos T25 de cultura confluente foram submetidos à irradiação com Co60, no laboratório de irradiação gama - LIG do Centro de Desenvolvimento de Tecnologia Nuclear - CDTN. 2 e 5 Gy foi aplicado. Após a exposição, as células retornaram à incubadora de CO2.

Cinética do tempo

As culturas *in vitro*, submetidas a 2 e 5Gy, foram mantidas em incubadora a 5% CO2, 37 ° C após irradiação, conforme figura abaixo. Alíquotas de células irradiadas e não irradiadas (controle) foram coletadas nos tempos atuais de 24, 48 e 72h pós-irradiação, respectivamente.



As culturas celulares foram mantidas em meio de cultura RPMI-1640 suplementado com 10% de soro fetal bovino e antibióticos gentamicina (50 µg.µL^{-1}) e estreptomicina (500 mg.mL^{-1}),

Em frascos de cultura T-25

Atmosfera úmida contendo 5% de CO_2 a 37°C.

Figura 3.3- Condições de manutenção das culturas nos ensaios *in vitro.*

3.3 - Avaliação de viabilidade – Teste Metil Tiazol Tetrazólio – MTT

Em amostras triplicadas, 100 µL de alíquotas de células de 24, 48 e 72h após a irradiação foram semeadas em cada poço de placas planas de ELISA (12 × 8) e mantidas em incubação por 8h. Um valor de 20µL (5 mg. ML-1) de MTT foi

adicionado a cada poço e as amostras foram devolvidas à incubadora, onde células viáveis metabolizaram o MTT por 4 h. Após o metabolismo, 80 µL foram descartados e adicionados 80 µL de isopropanol (0,04 M) em cada poço. As placas foram incubadas por mais 6 h. A viabilidade celular foi avaliada medindo a densidade óptica (DO) em leitor de ELISA no comprimento de onda de 595 nm. A primeira linha corresponde ao branco do leitor ELISA. As placas foram lidas com comprimento de onda de 595 nm em aparelho ELISA Elx800. O ensaio MTT quantificou a viabilidade celular e a proliferação celular com base na clivagem dos sais de tetrazólio (MTT). Após a incubação das células, uma solução de corante foi montada, cujo OD é medido pelo leitor de ELISA, e então a absorbância foi correlacionada com o número de células.

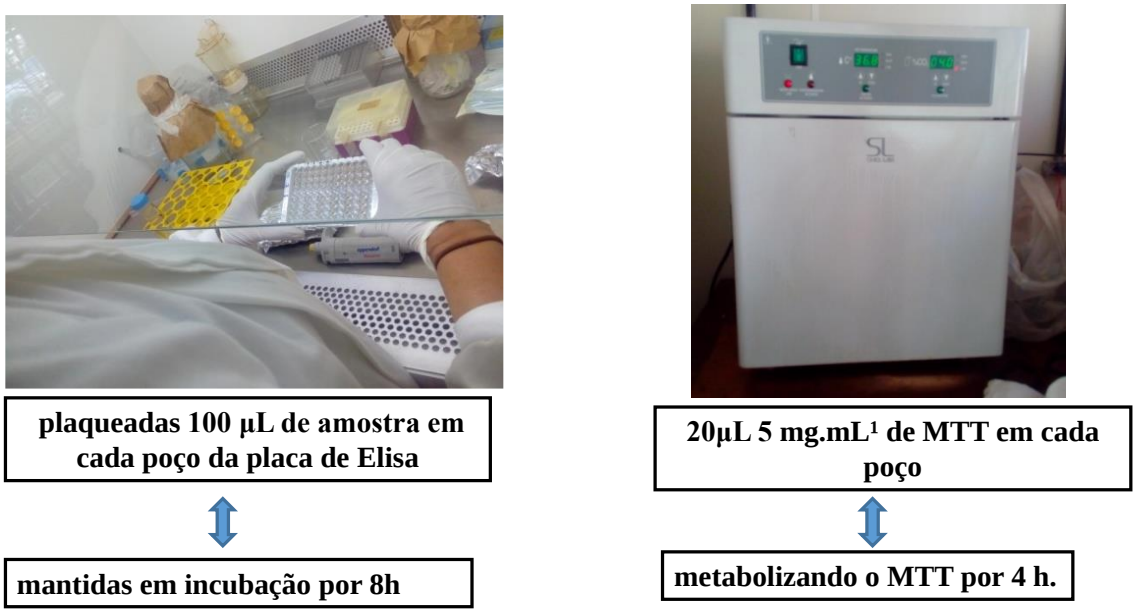
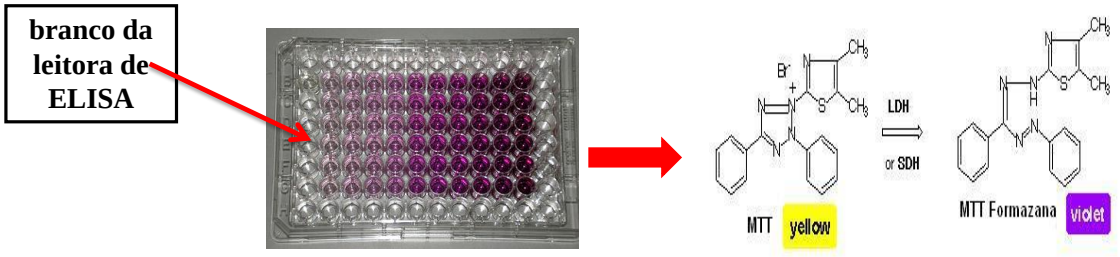


Figura 3.4 – A figura mostra a sequência básica de procedimentos para realização do ensaio de metabolização do sal tetrazólio (MTT).



❖ **Figura 3.5** – A figura mostra o principio do Teste colorimétrico pelo Metil Tiazol Tetrazólio – MTT, com quantificação da proliferação celular baseada na

clivagem do sal tetrazólio (MTT), no qual é possível correlacionar a absorbância com o número de células.

3.4- Teste Imunoenzimático (ELISA)

O teste de ELISA baseia-se em reações antígeno-anticorpo detectáveis por meio de reações enzimáticas (teste imunoenzimático). Basicamente**, o** anticorpo é impregnado à placa, o antígeno se liga à ele, posterirormente é adicionado outro anticorpo ligado à enzima para se ligar em um outro epítopo do antígeno e formar o complexo.

Citocina IL-6 - As concentrações de citocinas nos sobrenadantes foram medidas por ELISA em sanduíche de dois locais. As placas foram revestidas durante a noite com anticorpos monoclonais anti-IL-6 e incubadas com concentrações padrão de IL-6 recombinante ou sobrenadantes. Os anticorpos monoclonais anti-IL-6 foram usados, e as placas foram desenvolvidas usando anticorpos anti-NIP conjugados a rádio-peroxidase e ABTS (ácido 2,2-azino-di [3-etilbenztiolil] sulfônico) (Zymed, San Francisco, Substrato CA, EUA). A absorvância foi medida a 490 nm usando o bio-rad ELISA Reader (Enzyme-linked Immunosorbent Assay). A sensibilidade do ELISA foi de 20 pg mL-1.

Citocina TGF-β - A concentração nos sobrenadantes foi avaliada quanto aos níveis de TGF-β por ensaio ELISA em sanduíche (Quantitative Human TGF-β; R&D Systems, Inc., Minneapolis, EUA) de acordo com as recomendações do fabricante. A absorbância da solução produzida também foi medida a 490 nm. A absorbância foi diretamente proporcional à quantidade de TGF-β presente na amostra. Uma curva padrão foi montada traçando o valor médio de absorbância medido para cada padrão versus sua concentração correspondente.

ELISA - Enzyme-Linked Immunosorbent Assay

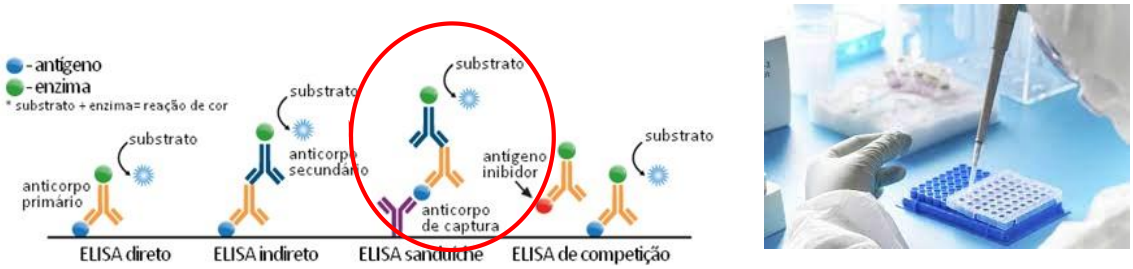


Figura 3.6- A figura mostra de forma esquematizada a sequencia de procedimentos para detecção de anticorpo.

Análise estatística

Foi realizada comparação intragrupo, de acordo com as duas doses de 2 e 5Gy e as diferentes concentrações dos extratos de Própolis. O teste t-Student foi utilizado para obter a diferença significativa entre os valores médios obtidos nas leituras. Foi adotado nível de significância de 5%. º

CAPITULO 4

THE PROPOLIS EXTRACT OF *TETRAGONA CLAVIPES* IS ABLE TO INHIBIT IL-6 AND TGF-β PRODUCED BY MDAMB-231 CELLS IRRADIATED WITH LOW-DOSE CO-60

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The investigation of the levels of interleukin 6 (IL-6) and TGF-β from *in vitro* breast adenocarcinoma MDA-MB-231 cells was performed to low radiation dose, in presence of *Tetragona clavipes* Propolis Extract (TCP) using Cobalt-60, at 2Gy dose, equivalent to a radiation therapy fraction, in the following conditions: non-irradiated (NIR) and irradiated (IR) samples with 0%, 1% and 10% TCP concentrations. Control and irradiation samples were tested at 24, 48 and 72 h. The optical density response in

MTT assay also was evaluated and morphological analysis was performed. The soluble IL-6 and TGF-β cytokines in the supernatants were measured by two-site sandwich ELISA. The analysis showed expansion at low dose rate after 48–72 h after radiation showing its radio resistance behavior at irradiation condition. The expressions of IL-6 and TGF-β in the supernatant on culture irradiated at 2 Gy were demonstrated. However, a significant decrease of IL-6 expression at 48 and 72h was observed in presence of 10% TCP. There is a possible potential recovering of the MDAMB cell viability at 2Gy at 48 and 72h after radiation exposition. The expression of IL-6 and TGF-β in breast MDAMB cells may be modulated by the presence of TCP.

4.1 INTRODUCTION

The TGF-β growth factor can induce the *in vitro* production of α-actin of the smooth muscle (α-SMA) in mammary fibroblasts, the main marker of myofibroblasts, and, therefore, can differentiate these cells in active fibroblasts.[1] This is one of the main modified pathways present in tumors, including breast cancer. [2,3,4] A crucial step in establishing metastases is the acquired cellular capacity of migration and invasion of healthy tissues. The essential and initial molecular process that leads to the tumor invasion is the epithelial-mesenchymal transition (EMT).[1] During this process, the cells lose their epithelial morphology, and functional properties, as cell polarity and normal cell-cell contact. In addition, the cells acquire mesenchymal properties, as similar morphology of the fibroblasts, the invasion proprieties, and start expression of mesenchymal markers, including N-cadherin and vimentin.[2] Among the mechanisms that induce this tumoral cellular transition, it is worth mention the expression of multiple extracellular promoters and the activation of intracellular signaling pathways, including the oncogenic signaling, cadherin Wnt3/β signaling, the increases of the reactive species of oxygen (ROS), as well as the damages in DNA. [5,6,7]

Like TGF-beta, IL-6 is also a growth factor that plays important roles in the growth and survival of the tumor cells [8]. In normal circumstances, IL-6 induces the differentiation of the serum cells; while, in the pathogenesis of some types of tumors, IL-6 cause proliferation and inhibits cellular apoptosis. [9]

IL-6 is a multi-functional cytokine that plays a basic role in the innate and acquired immune response, in hematopoiesis, in the inflammation, and in the regulation of the growth and differentiation of the cancerous cells.[8] The breast cancer expresses high levels of IL-6 in comparison with normal equivalent tissues. In addition, these levels increase with the histologic classification of the tumor. [10, 11] Some studies had shown the significant role of IL-6 in the migration and invasion of breast cancerous cells, as well as its epithelial transition for mesenchyme. [12, 13] Recently, Chang *et al.*, 2013, had shown increased levels of IL-6 at the edge of invasive breast tumors.[13]

It is worth standing out the role of the IL-6 as an activator of the pathway of signal transduction and activator of transcription 3 (STAT3) in some types of breast tumors. [14, 15] The activation of STAT3 can occur through the autocrine expression of IL-6, activating the paracrine effects from stroma.[16] STAT3 controls the expression of some genes related to cancer, both suppressors of tumor and oncogenes. Most of these genes are common in the healing of wounds in cancer.[17] Therefore, STAT3 activation constitutes an important linking between the inflammation and cancer.

The radiation therapy (RT) makes the malignant cells to lose its clonogenicity, probably by the inhibition of the pathways of cellular signaling, and, in contrast, preserves the functions of healthy tissue. RT presents great efficiency in the control of the tumors, using external radiation from X-ray beams. The primary gamma-radiation interacts with the tissue and produces electrons secondary that ionize the medium and create free-radicals. Such primary radicals interact with oxygen and the free radicals themselves, producing secondary radicals. Those radicals spread out in the overall cell and can provoke the rupture of the DNA chains. The presence of free radicals can lead the cell to death, through various mechanisms, or to inactivate the vital cellular systems

disabiliting the clonogenic reproduction.[17] However, studies have demonstrated that the irradiation of breast tumor with low doses and dose rate can induce radioresistance. It was already possible to demonstrate that the MDA-MB-231 is radioresistant at Co-60 low energy transfer (LET) radiation-type. [18, 19] No significant differences in the cellular viability for the MDA-MB-231 adenocarcinoma lineage after 2 Gy of irradiation in Co-60 was found in comparison to a non-irradiated control, suggesting a radioresistance to low dose and dose rate. Indeed, not only dose, but also the dose rate, as already demonstrated by Falcão and cols, 2015, possess clinical relevance, being able to reduce the tumor control in breast cancer in cobalt therapy.[19] It is worth mention that the success of the tumor control depends not only on the choice of the radiation therapy, but also on the robustness of immune response of the patient, including the intrinsic capacity of controlling the number of trigger signals started by the tumor, responsible for the maintenance of its clonogenicity.

The flora has natural substances with antioxidant properties, as endogenous enzymes (glutathione, superoxide dismutase, catalase) or hormones (melatonin), vitamins (C and E vitamins), carotenoids and phytochemicals (flavonoids, Curcumin). These compounds are often toxic in the doses required for radiation protection, which limits the clinical use. [20, 21] Propolis is a resinous substance collected by bees from different plant parts and is used to seal any openings in the hive and to eliminate possible invaders. It is known to contain a variety of chemical compounds as steroids phenolic acids, esters of phenolic acids, flavonoids and terpenoids, such as CAPE and Artepillin C. [22] Propolis possess biological active substances, including antibacterial, antiviral, antioxidant, anticarcinogenic, and anti-inflammatory effects. Propolis in conjunction of RT provides a very measurable protection against DNA damage caused by ionizing radiation in leukocytes during radiotherapy treatment. In addition, the immune activity provided using Propolis and related compounds increase hematopoietic regeneration and survival after radiation exposure.[23] Research on the effect of Propolis has increased and the knowledge of its property has been studied extensively in vitro and *in vivo* over time [24].

This paper addresses the role of this adjuvant natural substance in inducing signaling processes of a radioresistant cancer lineage exposed to radiation. The main goal was to search for the possible modulation of the soluble expressions of cancerous breast cells, in special TGF-beta and IL-6, in conditions of irradiation and in the presence of the aqueous extract of *Tetragona clavipes* Propolis.

4.2 MATERIAL AND METHODS

Aqueous Propolis Extract (Ext) – The aqueous extract of Propolis was obtained from the Fazenda Poranga located in the Municipality of Itacoatiara, State of Amazonas - Brazil, from the beehive of the *Tetragona Claviceps* species. The Propolis sample was kept under refrigeration at 4 °C. A quantity of 500 mL of deionized water and an amount of 100 g of crushed crude Propolis in the blender were added in a Becker and stored for 24 h. The mixture was heated in controlled temperature in a water bath at 80 °C for 2 h. Subsequently, this solution was disturbed in a blender and filtered through a funnel dripping in a conical flask. The filtrate was dried in a water bath for 48 h. Finally, the powder was resuspended in RPMI. The prepared extract concentrations were 1 % and 10 %, respectively.

Pre-established lineages. A breast adenocarcinoma MDA-MB-231 lineage was harvested. Cell samples derived from human breast invasive ductal carcinoma that were frozen and held in liquid Nitrogen were grown in Dulbecco's modified Eagle's medium (DMEM-SIGMA Chemical Co., St. Lois, MO, USA) supplemented with 10 % fetal bovine serum and Gentamicin (50 $\mu g\ \mu L^{-1}$) and Streptomycin (500 $mg\ mL^{-1}$) antibiotics (SFB - Cultilab, Brazil). Cells were maintained in T-25 culture flasks in 5 % CO_2 atmosphere at 37 °C. All cell manipulation was performed in a laminar flow hood. Cell growth was monitored under an inverted phase contrast microscope and the medium was replaced at every 24 h, according to cellular metabolism. Upon reaching the confluence, the cells were collected, and then sub-cultured. The cells were harvested every 24 h, with the respective replacement of the DMEM medium, supplemented with antibiotic-antimycotic, avoiding the saturation of the same.

Group controls. The groups were prepared with MDA-MB-231 culture flasks in triplicate following the conditions: non-irradiated and non-extract (Ext) incubation group; irradiated control group; and, irradiated and Ext incubated group. All four groups, with triplicate samples, were kept in culture as previously described. Both irradiated and non-irradiated (without extract) were control groups since those were not incubated with Teragona cavicles Propolis.

Radiation dose. The T-25 bottles containing the cells were sealed with parafilm and transported into a capped rigid plastic bottle, previously decontaminated with 70 % alcohol. The cells were irradiated in the LIG - Gamma Irradiation Laboratory of the Nuclear Technology Development Center in the *Centro de Desenvolvimento da Tecnologia Nuclear* - CDTN. The 4-cm tissue-equivalent slab plate was placed underneath the T25 flask, as well as the culture medium was filled up to 5-mm depth, in relation to the surface of cells attached. This procedure was necessary to achieve electronic balance and ensure the pre-defined doses in the adherent cells. The value of 2.0 Gy was applied in the irradiated groups. After exposure, the cells returned to 5% CO_2, 37 °C incubator.

Time kinetics. The *in vitro* cultures, exposed to 2 Gy, were kept in 5% CO_2, 37° C incubator after irradiation. Aliquots of supernatant in the flasks of all groups were collected at the times of 24, 48 and 72 h post-irradiation, respectively.

IL-6 Cytokine - The concentrations of cytokines in the supernatants were measured by two-site sandwich ELISA. The plates were coated overnight with anti-IL-6 monoclonal antibodies and incubated with standard concentrations of recombinant IL-6 or supernatants. The anti-IL-6 monoclonal antibodies were used, and the plates were developed using anti-NIP antibodies conjugated to radio-peroxidase and ABTS (2,2-azino-di [3-ethylbenzthiolyl] sulfonic acid) (Zymed, San Francisco, CA, USA)

substrate. Absorbance was measured at 490 nm using the bio-rad ELISA Reader (Enzyme-linked Immunosorbent Assay). The sensitivity of the ELISA was 20 pg mL^{-1}.

TGF-β Cytokine - The concentration in the supernatants was assayed for levels of TGF-β by sandwich ELISA assay (Quantitative Human TGF-β; R&D Systems, Inc., Minneapolis, USA) according to manufacturer's recommendations. The absorbance of the solution produced was measured at 490 nm, as well. The absorbance is directly proportional to the amount of TGF-β present in the sample. A standard curve was assembled by plotting the mean absorbance value measured for each standard versus its corresponding concentration.

Statistical analysis. The intragroup comparison was carried out, according to the two doses of 2 and 5 Gy and the different concentrations of Propolis extracts. The t-Student test was used to obtain the significant difference between the average values obtained in the readings. It was adopted a significance level of 5 %. The software used for the analysis was Origin 8 for Windows.

4.3 RESULTS

IL-6 Cytokine - The table 1 shows the effect of the aqueous extract of Propolis on the detection of cytokine Interleukin-6 (IL-6) in the culture supernatant of MDA-MB-231 cells, exposed and exposed to low dose (2 Gy) cobalt therapy, when treated and not treated with Propolis extract, at concentrations of 1 % and 10 %. The results showed a very similar detection of IL-6 in the supernatant of the non-exposed (control) culture and in the irradiated 2 Gy, after 48 h of irradiation, in consonance with the observations made in the MTT cell viability and in relation to the confluence of the cells observed in the culture flasks. The addition of 1 % Propolis extract to irradiated cultures appears to have had a significant effect on IL-6 production. However, when the irradiated MDA-MB-231 culture was treated with the 10 % aqueous Propolis extract, a rather significant ($p < 0.005$) decrease in IL-6 levels was observed compared to non-irradiated and

irradiated control and also to the culture that received the 1 % extract, suggesting that the Propolis could be probably acting on some pathway leading to the production of this pro-tumor cytokine. The data presents the expression of IL-6 in $pg.mL^{-1}$ determined by ELISA in *vitro* culture of MDA-MB-231 following the condition of irradiation and non-irradiated cells. Increasing the concentration of the extract at 10 % and exposing the cells to 2 Gy, the IL-6 concentration is lower than control.

Experimental group	Times (h)			Mean (Time)
	24	48	72	
Control: NIR	2.21 ± 0.02	3,95 ± 0.04	1,83 ± 0.009	2.66± 0.009
Control: 0% + 2 Gy	2.02 ± 0.01	3.71 ± 0.002	3.98 ± 0.011	3.24± 0.013
Extract 1% + 2 Gy	1.98 ± 0.011	3.87 ± 0.01	3.91 ± 0.01	3.25± 0.011
Extract 10% + 2 Gy	1.55 ± 0.007	1.15 ± 0.002	2.12 ± 0.007	1.61± 0.007

Table 4.1 – Detection of IL-6 determined by ELISA in *in vitro* culture of MDA-MB-231, non-irradiated and irradiated at 2 Gy dose and submitted to concentrations of 1% and 10% of aqueous Propolis extract (average values followed by standard deviations).

TGF-β Cytokine - Table 2 shows the effect of the aqueous extract of Propolis on the detection of Transforming growth factor-β (TGF-β) in the culture supernatant of MDA-MB-231 cells, exposed and exposed to low dose (2 Gy) to Co-60, when treated and not treated with Propolis extract, at concentrations of 1 % and 10 %. The results showed similar detection of TGF-β in the supernatant of the non-exposed (control) culture and in the irradiated 2 Gy, for the kinetics of 24, 48 and 72 h. With the addition of 1 % of the Propolis extract, a significant decrease in TGF levels was observed 72 h after irradiation. It is worth to mention that in the period of 24, 48 and 72 h there was a significant decrease in TGF-β levels, in the presence of the 10 % Propolis extract.

Groups	Times (h)			Mean (Time)
	24	48	72	
Control: 0% Ext, NIR	2.75 ± 0.007	3.82 ± 0.001	1,79 ± 0.006	2.78± 0.005
Control: 0% Ext + 2 Gy	2.02 ± 0.007	3.81 ± 0.002	2.02 ± 0.001	2.62 ± 0.007
Extract 1% + 2 Gy	2.78 ± 0.003	3.91 ± 0.002	1.51 ± 0.01	2.73± 0.003

Extract 10% + 2 Gy	1.54 ± 0.012	2.01 ± 0.002	1.12 ± 0.005	1.56± 0.005

Table 4.2 – Detection of TGF-beta determined by ELISA test *in vitro* culture of MDA-MB-231, before and after radiation exposure at 2 Gy and submitted to concentrations of 1 % and 10 % of aqueous Propolis extract. (average values followed by standard deviations).

4.4 DISCUSSION

The local control of a radioresistant tumor and the reduction of the chances of regional metastases in breast cancer are the main concerns in the radiation therapy, being a challenge of obscure success. Metastases results in a complex cascade of events whereby the cancerous cells leave the primary tumor and spread over distant organs proliferating and shaping secondary clonogenic focus. [25, 26] These events are regulated by a series of molecular factors, including growth factors and cytokines present on the microenvironment of the host tissues, or released by the cancer cell guests. [19, 25]

In the present study, the levels of TGF-β and IL-6, expressed by the radioresistant MDA-MB-231 cells after a low dose of radiation of low-LET, were significantly higher in comparison to the unirradiated control. The dose of 2 Gy induced the reduction of TGF and IL-6 after 24h; however, past 72 h, the cellular environment had recovered the control levels. At 72h, TGF-β and IL-6 levels presented no significant differences between the control and the irradiated samples. This fact represents an unfavorable situation, suggesting that the dose of 2 Gy is not efficient to keep the reduction of clonogênese tumoral in vitro in the irradiated conditions. These data are corroborated by Divella et al, 2013, that demonstrated the clinical impact of the serum

TGF-beta and CXCL1 chemokine, as predictive factors for clonogenesis and for the cell escape to the circulatory system, associating it to low prognostic of this illness.[27]

A high level of TGF-β seems to favor a more aggressive clonogenic phenotype promoting the growth of the tumor, extending the resistance to apoptosis, increasing the mobility of the cancerous cells and eventually metastases.[28] It is interesting to observe that, in some types of breast tumors, the *in situ* cellular surrounding tissue that compose the tumoral microenvironment, seem to be involved in the maintenance of a high level of TGF-β, through some specific processes of signaling.[29] In MDA-MB-231 culture, TGF-β was detected prominently at earliest 24 h in the supernatant and later observed a decline that can be associated to specific performances of some transcription factors. Considering that NF-κB and STAT3 pathways are chemotherapy targets in various types of breast tumors, it is possible to infer that they can mediate the regulation of TGF-β. [8] Hendrayane et al., 2014, had demonstrated an association between TGF-β1 and IL-6 since the TGF-β1 can mediate the negative regulation of IL-6. This observation was possible found through experiments with co-culture of isolated fibroblasts removed from histological sections of healthy breast tissue, surrounding the tumor. Therefore, it is worth mentioning the relevance of reproducing the most as possible the tumoral microenvironment in the in vitro assays. On the other hand, the modulation of the tumor targets, as cytokines or chemokines, to extend the control of the oncologic illness and to reduce relapse,[30] and may regulate the intensity and duration of some clinical specific responses, as well as may alter the processes of cellular conscription in favor of cancer.[31]

The use of natural substances becomes attractive when they hold the potential to modulate the cytokines in the tumor environment. In special, it is worth investigating the natural substances that can act and modulate the tumoral targets, with the potential coadjuvant role in the radiation therapy of radioresistant tumors, as the MDA-MB-231 cellular case. The aqueous Propolis extract may have held this role in the assays submitted to 2 Gy of irradiation from Co-60. The TGF-β and IL-6 in the supernatant of the MDAMB231 cultures were significantly affected in the group that received 10 % of

the extract, suggesting that the Propolis can be associated with the inhibition of these two pro-clonogenic co-factors in the in vitro assays.

Studies on the regulation of the clonogenesis of MDA-MB-231 cells have boarded the role of the IL-6 in the modulation of p16, p21, and p53 (proteins suppressors of oncogenes) and in the cellular differentiation of fibroblasts to myofibroblasts. It is well known that the anti-IL-6 monoclonal antibodies have influenced the IL-6 expression positively.[32, 33] On the other hand, the addition of recombinant IL-6r to the cultures of the radioresistant MDAMB lineage was capable to reduce the levels of expression of mRNA for these three tumor suppressor proteins, suggesting the regulation of the paracrine effect of MD-AMB-231 on the expression of oncogenes.[8] Studies showed that IL-6 actives the JAK/STAT3 pathway, and this route is capable to suppress p21 and p53 in fibroblasts in the stroma in the early 24 h in culture. Moreover, the molecular mechanism of suppression of p16, p21, and p53, dependent on STAT3, seems to involve another protein, the AUF1, whose modulation of these suppressor genes occurs post-transcription. [34-37]

As perspective, it is needed to evaluate if the effect of the Propolis extract will be pertinent on the pathways of the transcription of the tumor suppressor genes, through the manipulation of recombinant cytokines and monoclonal antibodies anti-IL-6 and anti-TGF-β in culture. The expectation is not only to elucidate the proper radioresistance of MDA-MB-231 lineage, but also to understand the role of the *Tetragona clavipes* Propolis in the negative regulation of protumoral cytokines. Such information will give support to a natural treatment in co-adjuvance to RT in breast cancer.

4.5- CONCLUSIONS

The Propolis extract of *Teragona clavipes* can inhibit IL-6 and Tgf-β produced by MDA-MBm-231 cells after exposition with low-dose of Co-60.

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CAPÍTULO 5

IN VITRO RADIATION PROTECTION OF PERIPHERAL BLOOD MONONUCLEAR CELLS BY *TETRAGONA CLAVIPES* PROPOLIS

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Background: Breast cancer has found notable importance in recent years, bringing serious concern on the public health policies due to its high incidence and mortality, especially in Brazil. Limitations due to toxic effects on normal tissues and changes in the immune system have often been present in the breast cancer radiotherapy and chemotherapy. It is promising the drug development of vegetal origin that induce immune system protection in patients submitted to radiotherapy and chemotherapy, if such compounds inhibit the depletion of the circulating cell number.

Objectives: This study aimed to evaluate the in vitro effect of the extract of *Tetragona clavipes* Propolis in the viability of the human peripheral blood mononuclear cells (PBMC).

Methods: The non-irradiated control (GC), irradiated control (GCI) and radiated and exposed to *Teragona clavipes* Propolis (GIE) (n=3) groups were established. The cells were irradiated with

low Linear Energy Transfer (LET) with predetermined doses of 2 and 5Gy, supplemented with Propolis extract in aqueous solution at concentrations of 0.5%, 1%, 5% and 10%. Post-irradiated sample was collected and viability assay by MTT was carried out at the time of 24, 48 and 72h.
Results: A significant decrease of PBMC viability was observed after 2 and 5Gy. However, GIE showed an increase of cell viability, especially in the 5 and 10% concentrations of the extract incubated in culture, even after 5 Gy.
Conclusion: the findings showed that the aqueous extract of *Teragona clavipes* Propolis is an exogenous protective agent to in vitro irradiated PBMC. This study opens a relevant perspective on the role of Propolis use as an adjuvant agent in protecting the immune system of patients undergoing breast radiotherapy.

5.1 INTRODUCTION

Breast cancer has often grown a hormone-dependent malignant tumor, responsible for most of the cancer-related mortality in women. While advances in treatment and prevention of breast cancer have emerged over the last decade, the phenomenon of multiple drug resistance has been the main causes of the aggravation of this morbidity as a reflection of the radiotherapy and chemotherapy failures (Aller et al. 2009).

Radiotherapy represents the main part of the primary conservative treatment of breast carcinoma, holding the goal of a better in situ tumor control (Zucali, 1992; Liu, 2009). According to Perez et al. (2015), conformational protocols, assembled in the 3D planning system for the treatment of breast cancer, consider that the breast exposure should be carried out in two opposite tangential fields in multiple fractions of 1.8 to 2.0 Gy daily, five days a week, accumulated up to 45 to 50 Gy. Deep fields, expanding the radiation portals, cover large part of the chest, mediastinum, and lung. It can be indicated in patients with lymph node involvement. Such fields can also include the irradiation of the internal mammary chain, when taken in addition from the medial border 1, 3 or 4 cm beyond the median line. Radiotherapy patients submitted to large fields and treated with cytotoxic chemotherapy have often compromised the immune system, and cell blood monitoring is required, and it limits the therapy.

Drug development of vegetal origin that induce radiation protection to the immune system of patients in radiotherapy is promising, considering that such compounds may inhibit cell phenotypic change and depletion of the number of circulating cells. Therefore, it is important to identify non-toxic compounds, effective and of low cost that can serve as immune protection in patients submitted to cancer treatment to prevent opportunistic infections.

Many synthetic compounds are being tested and have a particular interest but have been a limited clinical success. These substances have antioxidant properties that occur naturally, as endogenous enzymes (glutathione, superoxide dismutase, catalase) or hormones (melatonin), vitamins (C and E vitamins), carotenoids and phytochemicals (flavonoids, Curcumin) (Weiss & Laudauer 2009). These compounds are often toxic in the doses required for radiation protection, which limits the clinical use (Vijayalaxmi et al. 2004; Shirazi et al. 2007). Propolis is a resinous substance collected by bees from different plant parts and is used to seal any openings in the hive and to eliminate possible invaders (Santos, 2011). It is known to contain a variety of chemical compounds as steroids phenolic acids, esters of phenolic acids, flavonoids and terpenoids, such as CAPE and Artepillin C (Huang et al 2014).

Adjuvant herbal medicines, including Propolis, have been described in the literature and have shown their efficiency limiting the tumor clonogêneses or as radioprotective and nontoxic agent. The main chemical constituent present in Propolis is the Caffeic acid phenethyl ester (CAPE). It is proven that this compound possesses biological activities, including antibacterial, antiviral, antioxidant, and anticarcinogenic and antiinflammatory effects (Park et al. 2009). Propolis supplementation with radiotherapy treatment provides a very measurable protection against DNA damage caused by ionizing radiation in leukocytes of patients during radiotherapy treatment (Ebeid et al 2016). In addition, the immune activity provided using Propolis and related compounds increase hematopoietic regeneration and survival after induction by radiation (Orsolic et al. 2007).

Research on the radioprotective action of Propolis has increased and the knowledge of its property has been studied extensively in vitro and *in vivo* over time (Benkovic et al. 2009). Propolis prescription becomes an interesting approach as adjuvant therapy in the modulation of cellular chemotaxis associated with radiation, in the treatment of hypoxic tumors. The monitoring of the size of the tumor, as well as inhibition of nocireceptors it is essential to determine the prognosis of the treatment (Potti et al. 2002).

The present in vitro study proposes to investigate the role of the extract of *Tetragona clavipes* Propolis on the viability of the human peripheral blood cells (PBMC), exposed to two doses of ionizing radiation.

5.2 METHODS

Preparation of the Propolis extract. Raw Propolis was obtained from the Poranga farm in the municipy of Itacoatiara at Amazonas State, Brazil, from the hive of *Tetragona clavipes* bee specie. The sample of Propolis was kept cool under -4°C. Subsequently, the extract of Propolis was prepared, as described by Matsushige et al., 1996, with modifications. In a Becker, it was added 500mL of purified water and 100 g of raw Propolis crushed in blender and held by 24h. The mixture was heated at a constant temperature water bath at 80°C for 2h. Subsequently, this solution was mixed in Blender, filtered (gauze filter), and coupled with a funnel and a kitasato. The filtrate was dry in water bath during 48h. Finally, the resulting powder was ressuspended in RPMI medium in the experimental concentrations.

Pre-established lines and in vitro culture maintenance. PBMCs obtained from healthy volunteers were set in the culture. Cell cultures were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotic gentamicin

(50μg.μL^{-1}) and streptomycin (500mg.mL^{-1}), in T-25 culture bottles in humid atmosphere containing 5% CO_2 at 37°C.

Separation of PBMC. The PBMCs of patients were separated according to the procedure described by Gazzinelli et al., 1983. The heparinized blood was applied in 15mL tubes, siliconized, containing a mixture of Ficolldiatrozato (Organon Teknika Corporation; Durham, NC), in the proportion of one part of Ficoll-diatrozato to two parts blood. The solution was subjected to centrifugation for 30min, in 1,400rpm at room temperature. At the end of the spin cycle, a ring of mononuclear cells at the interface between the Ficoll and the plasma was observed. It was carefully removed with the aid of a Pasteur pipette and transferred to sterile, taped 15mL tubes (Falcon No. 2070). The 15mL volume was completed by RPMI-1640 solution (Gibco®) not supplemented and was submitted to another centrifugation (10min, 1,200rpm). The cells were washed twice (10min, 1,200rpm). Finally, an aliquot of cell suspension was collected and diluted (1:20) at Eppendorf tube containing 90μL of Turck solution and the cell number was determined by Neubauer Chamber count, in an optical microscope. The cell concentration was adjusted to a suspension containing 1.0×10^6 cell.mL^{-1} with RPMI-1640 supplemented. All manipulation of the cells was performed under sterile conditions, in the laminar flow cabinet (Biological Cabinet BBL 60474 model).

Group controls. The two control groups were prepared with PBMCs in a condition of non-irradiated and non-water Propolis extract presence, kept in culture as previously described.

Radiation dose kinetics. T25 bottles containing PBMC were sealed with parafilm and transported inside a rigid capped plastic bottle, previously decontaminated with 70% alcohol, to the location of irradiation. A 4-cm tissue-equivalent slab plate was placed underneath the T25 bottle, as well as the culture medium was filled up to 5-mm high, in relation to the surface of cells attached. This procedure was necessary to achieve electronic balance and ensure the prescribed doses in the adherent cells. T25 bottles of confluent culture underwent irradiation with Co60, in the laboratory of gamma irradiation – LIG at the *Centro de Desenvolvimento da Tecnologia Nuclear* – CDTN. 2 and 5 Gy was applied. After exposure, the cells returned to CO_2 incubator.

Time kinetics. The in vitro cultures, subject to 2 and 5Gy, were kept in 5% CO_2, 37° C incubator after irradiation. Aliquots of irradiated and non-irradiated cells (control) were collected in the present times of 24, 48 and 72h post-irradiation, respectively.

Viability assessment. In triplicate samples, 100 μL of cell aliquots of 24, 48 and 72h after-irradiation were plated in each well of ELISA flat-plates (12 × 8) and kept in incubation for 8h. A value of 20μL (5 mg. mL^{-1}) of MTT was added to each well and the samples were returned to the incubator where viable cells metabolized the MTT for 4 h. After metabolism, 80 μL were disposed and added 80 μL of isopropanol (0.04 M) in each well. The plates were incubated for more 6 h. Cell viability was evaluated by measuring the optical density (OD) in ELISA reader at 595nm wavelength. The first row corresponds to the white of the ELISA reader. The plates were read with 595nm wavelength in ELISA Elx800 apparatus. The MTT assay quantified the cell viability and the cellular proliferation based on the cleavage of tetrazolium salts (MTT). After incubation of the cells, a dye solution was assembled which OD is measured by the ELISA reader, and then the absorbance was correlated with the number of cells.

Statistical analysis. Intragroup comparison was carried out, according to the two doses of 2 and 5Gy and the different concentrations of Propolis extracts. The t-Student test was used to obtain the significant difference between the average values obtained in the readings. It was adopted a significance level of 5%. The software used for the analysis was SPSS for Windows 7.

5.3 RESULTS

Propolis extract concentrations of 0.5% and 1%. The quantitative evaluations of PBMC viability exposed to the extract of *Teragona clavipes* Propolis in the 0.5 and 1% concentrations were depicted in Fig.1. The data were presented in the collected time kinetics of 24, 48 and 72 h after extract incubation. It has been demonstrated that viable cells could form crystals of formazan product for the cleavage of tetrazolium salt. Considering the time interval of 72h, in Figure 1, the percentage of optical density increased significantly ($p>0.005\%$) in relation to: i) the groups from other time intervals; ii) the control group (not supplemented with Propolis); or, iii) the group that received lower concentration of Propolis.

Concentrations of the Propolis extract of 0.5%, 1.0%, 5.0% and 10%, without radiation. Fig.2 illustrates the profiles of cellular viability of PBMC after incubation to the propolis in the concentrations of 0.5, 1.0, 5.0 and 10%, in comparison with PHA at 1.0μg.mL^{-1}. It was observed that the extract of propolis at 10% has a similar effect to the PHA. There are significant statistic differences between the control and the groups incubated in the concentration of 1, 5 and 10% of propolis.

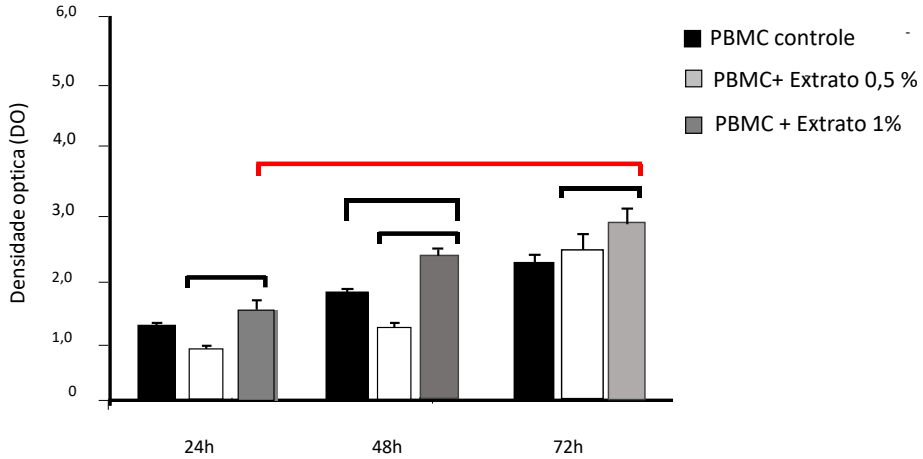

Figure 5.1 - Survival kinetic profiles in time of 24, 48 and 72h, determined by the MTT assay on PBMC in vitro culture before and after incubation of 0.5% and 1% concentrations of water extract of Propolis. The horizontal bars represent statistically significant difference ($p<0.05$) between the average values of three wells samples, containing PBMCs treated previously with

the extract compared to PBMC control free of extract. The red bars represent groups with statistically significant difference ($p<0.05$) in the time kinetics of 24, 48 and 72h.

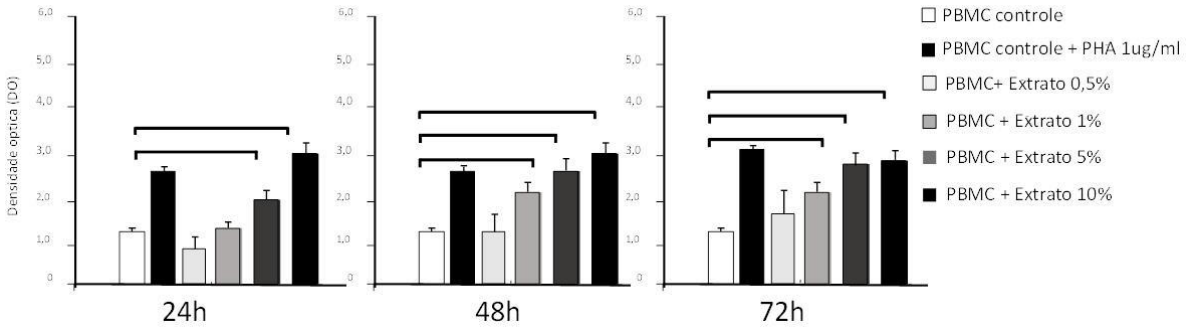

Figure 5.2 - Survival kinetic profiles in time of 24, 48 and 72 h, determined by the MTT assay on PBMC in vitro culture before and after incubation of 0.5%, 1%, 5% and 10% concentrations of aqueous extract of Propolis. The horizontal bars represent statistically significant difference ($p<0.05$) between the average values of three wells samples, containing PBMCs treated previously with the extract compared to PBMC control free of extract.

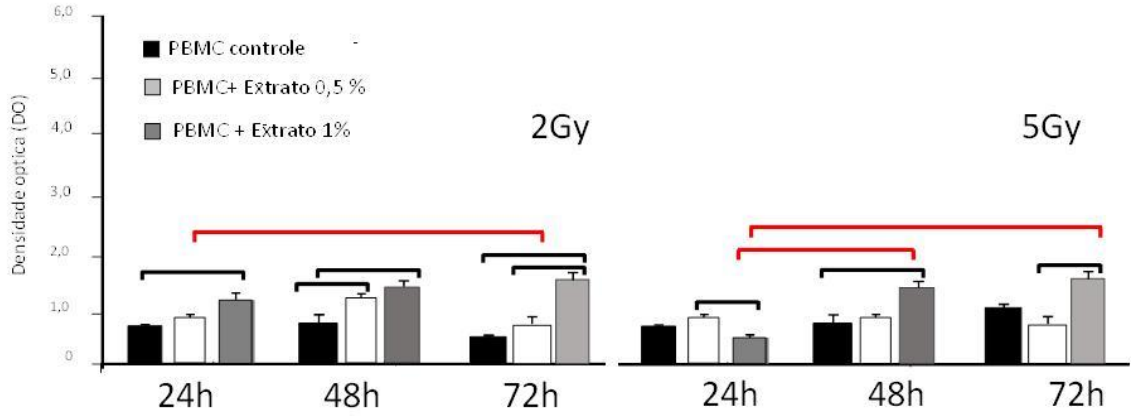

Figure 5.3 – Survival kinetic profiles in time of 24, 48 and 72h, determined by the MTT assay on PBMC in vitro culture before and after incubation of 0.5% and 1% concentrations of aqueous extract of Propolis, exposed to 2 and 5Gy. The horizontal bars represent statistically significant difference ($p<0.05$) between the average values of three wells samples, containing PBMCs incubated previously with the extract compared to PBMC control free of extract. The red bars represent a statistically significant difference ($p<0.05$) in the time kinetics of 24, 48 and 72h.

Propolis extract with 0.5% and 1% concentrations following 2 Gy and 5Gy. Figure 3 depicted the profiles of the average values of the PBMC viability subjected to combined agents: irradiation and Propolis extract.

The data from nonirradiated control groups, without and with Propolis incubation, presented a genotoxic damage lesser than those from irradiated group, in lower concentrations of 0.5% associated with 2 and 5Gy as in 1% associated with the same doses of radiation.

Fig.3 shows the profiles of cell viability assessed by MTT assay for PBMCs in vitro, from homogenized cell suspension in T25 bottle, irradiated by 2Gy. The data show a significant decrease ($p<0.005$) of the cell survival at 12h without Propolis incubation in relation to cell cultures supplemented with Propolis extract. It is interesting to note that at the 12h up to 72h time there was no significant increase of cell proliferation in cell cultures that were submitted to the 0.5% Propolis concentration, over twice in proliferation from the 1% concentration.

As shown in Fig.3, the PBMC viability in the three groups of cell cultures who received only radiation was significantly reduced ($p<0.005$). In relation to the cell viability in groups that received 2Gy, and exposed to the Propolis extract, the cellular viability was significantly higher than the levels of the control ($p<0.005$). Considering the pre-established time intervals, no significant difference was observed in the cell growth that received radiation plus Propolis incubation.

5.4 DISCUSSION

The present study has investigated the influence of Brazilian Propolis, particularly from Itacoatiara, Amazonas State, on cytotoxic damage and the proliferative potential of PBMC cells irradiated with Co60.

Although the exact mechanism of action of Propolis on radiation protection is not fully elucidated, several studies have shown that the pharmacological properties of Propolis are attributed mainly to the presence of flavonoids due to their action as scavengers of free radicals (Rithidech et al. 2005). Such scavengers of free radicals are likely to have key role in radiation protection, since ionizing radiation induces toxicity which is measured primarily by free radicals and its action in DNA (Montoro et al. 2011). According to Ebeid et al., 2016, Propolis supplementation with radiotherapy treatment provides a very measurable operation against DNA damage by ionizing radiation in leukocytes of patients. In addition, Propolis has beneficial effects on serum antioxidant capacity and improves digestive use of iron and hemoglobin regeneration efficiency.

Aqueous extract of Propolis stimulated PBMC cell growth at both 2 and 5Gy doses (Figure 4). The combined treatment with Propolis (1%) and radiation stimulated cell growth by inhibiting the cytotoxic effect of radiation especially after incubation for 72 h. The results showed that the extract of Propolis on the 1% concentration, introduced radiation protective effect on PBMCs against the damage caused by the interaction of ionizing radiation with its biological components. Ionizing radiation is widely used for the treatment of breast cancer. However, one of the limitations of the radiation use is its toxic effects on normal tissues. Human blood cells showed radio sensitivity in all gamma-rays from Co60 doses (Mays et al. 2015). Through these data, it can be said that Propolis showed a stimulatory effect on cell proliferation in PBMCs.

5.5 CONCLUSION

The association of Propolis and radiation exposure provided no significant decrease in the PBMC viability at 24, 48 and 72h. Thus, the Propolis of *Tetragona clavipes* offered a protection against damage caused by ionizing radiation in PBMC. This finding can serve as a basis for the potential use of Propolis in combination with radiation therapy, to protect immune circulating cells from genotoxic damage caused by radiation. Studies on the chemical constituents responsible for this radio-protective effect is needed to better characterize this radioprotector substance.

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CAPÍTULO 6

THE ROLE OF REGULATORY T CELLS, INTERLEUKIN-10 AND IN VIVO SCINTIGRAPHY IN AUTOIMMUNE AND IDIOPATHIC DISEASES – THERAPEUTIC PERSPECTIVES AND PROGNOSIS

REVIEW ARTICLE

Como já mencionado anteriormente, tem sido abordado pela literatura que o sucesso do tratamento do câncer pode estar diretamente conectado à interferência de fatores intrínsecos e extrínsecos ao organismo, que de fato, irão contribuir para o retorno da homeostasia (Pinheiro, 2007). Dessa forma, torna-se atraente e necessário elucidar questões concernentes ao comportamento do sistema imune diante estímulos extrínsecos, associados ou não aos tratamentos convencionais, como é o caso da própria radioterapia. Considerando como objetivo primordial do tratamento do câncer a redução e/ou morte das células tumorais, qual seria a contrapartida do organismo em relação ao sucesso do mesmo e os danos desencadeados pela radiação sobre células do sistema imune? Vale reportar que estudos têm demonstrado que a viabilidade de células do sistema imune altamente radio sensíveis é alterada em função de doses de recebidas (Falcão et, 2015).

É interessante nos reportarmos à capacidade de reorganização do sistema imune após a radioterapia, no tratamento do câncer, pode estar associada à substituição de populações celulares do SI que expressam fenótipos celulares "típicos" de quadros de imunossupressão, devido à radio sensibilidade. Entretanto, existem vários questionamentos bastante relevantes e ainda não elucidados sobre possíveis alterações desses fenótipos celulares, que poderiam estar conectados ao efeito potencial de terapias associadas à própria radioterapia. Recentemente nos reportamos à capacidade do sistema imune em expressar fenótipos celulares diferenciados, diante de desordens deste mesmo sistema, em patologias de origem autoimune e/ou idiopática (Falcão & Campos, 2017). Neste contexto, demonstramos através de estudo abaixo posto que as células do sistema imune, quando submetidas a estímulos, podem apresentar comportamento e alteração de fenótipo e serem moduladas por co-fatores liberados pela própria célula do SI (autólogos), como a interleucina 10, por exemplo. No capítulo, apresentaremos, na íntegra, nosso artigo de revisão que certamente poderá fornecer suporte para a hipótese de que o organismo humano seria capaz de alterar e adequar o fenótipo de suas células do SI, devido à modulação endógena desencadeada por terapias combinadas no tratamento de células tumorais *in vitro.* (Falcão et al, submetido 2019). Esse estudo estará sendo apresentado adiante, como parte dos resultados obtidos em nosso projeto.

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REVIEW ARTICLES

The role of regulatory T cells, interleukin-10 and in vivo scintigraphy in autoimmune and idiopathic diseases – Therapeutic perspectives and prognosis

O papel de células T regulatórias, da interleucina 10 e da cintilografica *in vivo* em doenças autoimunes e idiopáticas – Perspectivas terapêuticas e prognóstico

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SUMMARY

Previous studies have demonstrated the expression of the CD25 marker on the surface of naturally occurring T cells (T_{regs}) of mice, which have a self-reactive cellular profile. Recently, expression of other markers that aid in the identification of these cells has been detected in lymphocyte subtypes of individuals suffering of autoimmune and idiopathic diseases, including: CD25, CTLA-4 (cytotoxic T-lymphocyte antigen 4), HLA-DR (human leukocyte antigen) and Interleukin 10 (IL-10), opening new perspectives for a better understanding of an association between such receptors present on the cell surface and the prognosis of autoimmune diseases. The role of these molecules has already been described in the literature for the modulation of the inflammatory response in infectious and parasitic diseases. Thus, the function, phenotype and frequency of expression of the a-chain receptor of IL-2 (CD25) and IL-10 in lymphocyte subtypes were investigated. Murine models have been used to demonstrate a possible correlation between the expression of the CD25 marker (on the surface of CD4 lymphocytes) and the control of self-tolerance mechanisms. These studies provided support for the presentation of a review of the role of cells expressing IL-2, IL-10, HLA-DR and CTLA-4 receptors in the monitoring of immunosuppression in diseases classified as autoimmune, providing perspectives for understanding peripheral regulation mechanisms and the pathophysiology of these diseases in humans. In addition, a therapeutic approach based on the manipulation of the phenotype of these cells and ways of scintigraphically monitoring the manifestations of these diseases by labeling their receptors is discussed as a perspective. In this paper, we have included the description of experiments in ex vivo regulation of IL-10 and synthesis of thio-sugars and poly-sugars to produce radiopharmaceuticals for monitoring inflammation. These experiments may yield benefits for the treatment and prognosis of autoimmune diseases.

Keywords: Treg cells; IL-10; autoimmunity; idiopathies; scintigraphy

RESUMO

Estudos anteriores já haviam demonstrado a expressão do marcador CD25 na superfície de células T de ocorrência natural (T_{regs}) de camundongos, que apresentam perfil celular autorreativo. Recentemente, foi detectada, em subtipos de linfócitos de indivíduos acometidos por doenças autoimunes e de causa idiopática, a expressão de outros marcadores, que auxiliam na identificação dessas células, entre os quais: CD25, CTLA-4 (*cytotoxic T-lymphocyte antigen 4*), HLA-DR (*human leucocyte antigen*) e Interleucina 10 (IL-10), abrindo novas perspectivas para a melhor compreensão de uma associação entre esses receptores presentes na superfície celular e o prognóstico de doenças autoimunes. O papel dessas moléculas já havia sido descrito na literatura na modulação da resposta inflamatória em doenças infectoparasitárias. Dessa forma, foram investigados a função, o fenótipo e a frequência de expressão, do receptor de cadeia a da IL-2 (CD25) e de IL-10 em subtipos de linfócitos. O modelo murino tem sido utilizado para demonstrar uma possível correlação entre a expressão do marcador CD25 (na superfície de linfócitos CD4) e o controle dos mecanismos de autotolerância. Essas pesquisas forneceram suporte para apresentação de uma revisão sobre o papel das células que expressam os receptores de IL-2, IL-10, HLA-DR e CTLA-4 no monitoramento da imunossupressão, em doenças de classificação autoimune, abrindo perspectivas para o entendimento dos mecanismos de regulação periférica e sobre a fisiopatologia dessas doenças no ser humano. Além disso, é discutida como perspectiva uma abordagem terapêutica fundamentada na manipulação do fenótipo dessas células, bem como de modos de monitoramento cintilográfico das manifestações dessas doenças, por meio da marcação de seus receptores. Nestes, foram incluídas descrições das experiências em regulação *ex-vivo* de IL-10; de síntese de tioaçúcares e de poliaçúcares para produção de radiofármacos para monitoramento de inflamações. Essas experiências podem trazer benefícios na terapia e no prognóstico de doenças autoimunes.

Palavras-chave: células Tregs; IL-10; autoimunidade; idiopatias; cintilografia

6.1 INTRODUCTION

The immune system is orchestrated by a complex network of components interconnected by cells with their various receptors, secreted mediators, expressed molecules, activated biochemical pathways and other components that, together and at different anatomical sites, enable the body to respond to different antigenic stimuli.[1,2] Generally, the responses are triggered by the interaction of exogenous antigens with the antigen-presenting cells, strategically addressed and responsible for antigen capture, transport and processing. Often, the defense strategy becomes permanent, conferring an immunological memory, capable of ensuring better response efficiency in later exposures.[1,2]

With the diversity of potential antigenic exposures, only a highly adaptive immune system can distinguish and respond to the various antigenic sequences found. The rationale for recognition of this system is the product of random recombination of gene segments that generate lymphocytes with enormous receptor diversity. Such receptors characterize the cell phenotype of the lineage. The type and number of receptors are causally linked to the response to the different antigens (Ag).

The functions of the immune system include regulatory abilities, the mechanism responsible for ensuring that responses to antigens do not reach pathological and inhibitory levels, so that the immune system is not unduly activated against its own antigens, producing autoimmune disorders. Central tolerance is induced in the primary lymphoid organs, because of the recognition of autoantigens by immature T lymphocytes. To perform the proper protective function, multiple T-cell clones with wide antigen recognition diversity undergo a rigorous selection and thymic maturation process that occurs by recognizing their own peptides linked to major histocompatibility complex (MHC) molecules. The ability to distinguish between self and non-self-antigens is defined as immunological tolerance and is critical to avoid intense self-recognition that can lead to pathological autoimmune responses. Therefore, autoreactive thymocytes that recognize autoantigens with high affinity are eliminated by clonal deletion in the thymus.[3,4] While this is an efficient mechanism, it is known that some autoreactive cells can dodge this barrier and leave the thymus, and can be activated in the periphery with potential to generate autoimmunity. The fact that autoreactive cells

can be detected in the periphery clearly demonstrates that the thymic selection mechanism responsible for the elimination of autoreactive T cell clones is incomplete.[4,5] In this case, how can we ensure that such autoreactive cells will not be reactivated, promoting a break in tolerance and thus the emergence of autoimmune diseases? In other words, the immune system needs different, redundant features to ensure that potential autoimmune responses do not occur.

Peripheral tolerance mechanisms have been described in $CD4^+$ T cells and occur through anergy, clonal deletion and T cell suppression. Anergy may be induced during the Ag recognition process by T cells when: a) antigen presenting cells (APCs) do not express co-stimulatory molecules, thus rendering T cells incapable of responding to Ag; or b) when T cells express inhibitory receptors. In clonal deletion, there is repeated stimulation of T cells by antigens, resulting in cell death by apoptosis. The mechanism of suppression would be exerted by regulatory T cells (T_{regs}). T_{regs} represent a subpopulation of T lymphocytes characterized by the expression of $CD25^+$molecules and the nuclear factor *Foxp3*. The *Foxp3* factor induces suppression of effector T cells, blocking the activation and function of these lymphocytes, thus being important in the control of the immune response to self and non-self antigens.[3] The activation regulation can best be understood from Figure 1.

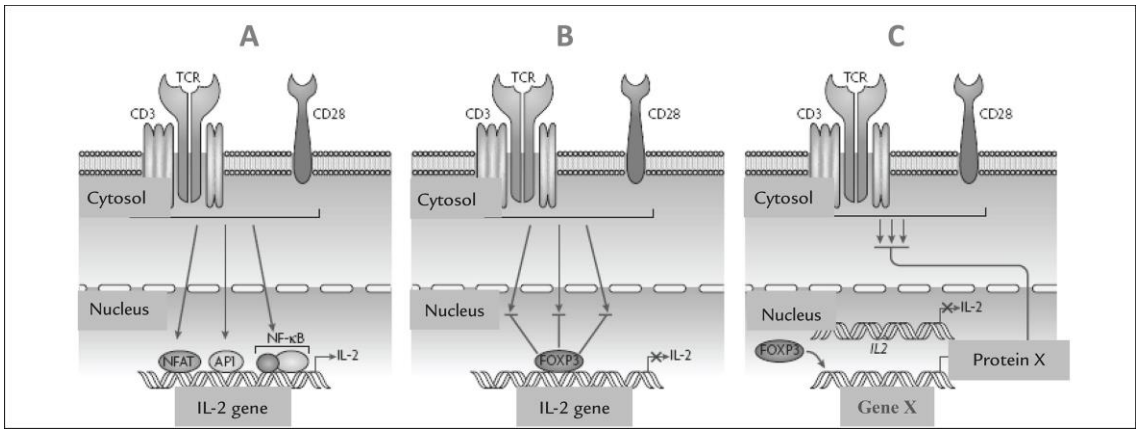

Figure 6.1- Regulation of T cell activation mediated by *Foxp3*. A. Signaling in effector CD4+ T cells. The binding of the T cell receptor (TCR) and the CD28 co-stimulatory molecule leads to the activation of the signaling pathways, resulting in the translocation of NFAT (nuclear factor of activated T cells) and AP1 (activator protein 1), with

subsequent transcription of the IL-2 (interleukin 2) gene. B. Model of direct regulation of TCR mediated by *Foxp3* signaling. In this model, the *Foxp3* factor blocks TCR signaling through the inhibition of activation mediated by NFAT, NF[5]-kB and AP1. C. Indirect regulation model of TCR signaling: *Foxp3* factor modulates TCR signaling through the expression of a factor that can inhibit TCR-induced signals. (Adapted from Campbell and Ziegler.[6])

It is possible that autoimmune disorders may be associated with failure to eliminate or inactivate high-affinity autoreactive cell clones during their ontogeny, and there may or may not be the failure of the immune system to control autoreactive intermediate affinity clones that have escaped to the periphery.[5,7] In this context, cells with a cellular response regulation function are fundamental and are also important in the modulation of the processes of eliminating pathogen and tumor antigen. These mechanisms occur with destruction of self-tissues, exposure of autoantigens and production of pro-inflammatory cytokines, which, unless regulated, favor the induction and maintenance of autoimmune events. To exercise their function, the fundamental property of T_{regs} is the ability to: i) produce cytokines with the cellular response modulating function of TGF-b; and ii) induce cell-cell contact-mediated suppression. These soluble substances act in a complex network of regulatory mechanisms designed to ensure the modulation of immunological responses to the various antigens derived from infectious agents, tumors, autoantigens, and allergens. Among T cells, several subpopulations exhibit regulation properties for exacerbated inflammatory response, such as IL-10-producing T_{regs}, which suppress some cytotoxic T cell responses in vivo,[5,7-10] including: CD8+CD28- T cells, $CD56^+$ T cells, gd T cells[11-13] and $CD4^-CD8^-$ T cells.[13] In addition to T cells, there are other cell subtypes that have been described with such properties. IL-10-producing $CD1^+$ B cells are among them.[14]

Particularly among T_{regs}, there has been a strong emphasis on naturally-occurring T cells ($CD4^+CD25^+$ T cells), as described by Sakaguchi et al.,[15] which are potentially capable of suppressing activation, proliferation and/or effector function of $CD4^+$and $CD8^+$ T cells, and, possibly, NK cells, NK/T, B lymphocytes and dendritic cells.[15]T_{regs} are indispensable for the maintenance of tolerance mechanisms and knowledge of their functions is fundamental for understanding the pathophysiology of autoimmune diseases and to subsidize the strategies of interference in the mechanisms

of recovery of tolerance in these pathologies. The need to review basic knowledge about $CD4^+CD25^+$ T cells, their role in different rheumatic diseases, and the prospects of advancement in the treatment of autoimmune diseases through the manipulation of these cells is therefore justified.

Naturally occurring T cells are related to the maintenance of self-tolerance and are very important for the maintenance of homeostasis of the immune system.[16] T_{regs} are involved in the inhibition of the activation and expansion of autoreactive lymphocytes in the peripheral tissues and present an inhibitory capacity with a proven role in the negative regulation of the immune response also against exogenous antigens and autoantigens.[17-19]Currently, T_{regs} have been investigated for their role in the immunomodulation of responses in inflammatory, neoplastic, autoimmune syndromes and also in transplant rejection, in the hope of opening other therapeutic perspectives to control exacerbated immune responses without the induction of anergy or nonresponsiveness, but by activating cellular function.[20-23] Early reports on cell subtypes specialized in regulating the immune response occurred in the 1970s, when it was shown that some subtypes of T lymphocytes were able to suppress the development of autoimmune diseases.[24] Later, other authors[1,15] demonstrated the constitutive labeling of the a-chain receptor of IL-2 (CD25) by $CD4^+$ T lymphocytes and attributed to it a role in the suppression of autoimmune diseases in mice. Proof of this role was possible by the removal of $CD25^+$ splenocytes in healthy rodents, triggering autoimmune disorders such as thyroiditis, insulinitis, polyarthritis, glomerulonephritis, and graft versus host disease. It was also demonstrated that adoptive transfer of this population inhibited autoimmunity in experimental models.[15,25]

Interest in the study of T_{regs} is due to the key function of this cellular population in the maintenance of the mechanisms of self-tolerance and in the regulation of the immune response.[20] $CD4^+CD25^+$ T lymphocytes represent 5 to 10% of total $CD4^+$ cells in peripheral blood.[25-27] Evidence obtained in later studies shows that $CD4^+CD25^+$ thymocytes are selected in the thymus from interactions with proper peptides presented by MHC-II molecules.[27] Positive selection of these cells depends on high-affinity interactions with autoantigens expressed on MHC molecules.[28] The mechanism by which $CD4^+CD25^+$ T cells escape negative selection is still controversial, but it is believed that these, once positively selected through high affinity

recognition of their own peptides, produce anti-apoptotic molecules that protect them from negative selection.[29] T_{reg} cells, besides the thymic generation, can be induced in the periphery by the action of specific soluble factors on naïve cells that have just left the thymus.

Annunziato et al.[30] evaluated phenotypic and functional characteristics of human thymus cells and have demonstrated that these cells respond to chemotactic signals from macrophages and epithelial cells constitutive of the thymus itself, and that are capable of expressing $CD4^+$, $CD25^+$ and mTGF-b1, as well as molecules directly with immunosuppressive function, such as CTLA-4. These cells had low production of IL-10 and none of IL-2, IL-4, IL-5, IL-13 and IFN-U.[30,31] In addition to thymus, human T_{reg} cells were isolated in other microenvironments, such as secondary lymphoid organs, e.g., tonsils and spleen, as well as umbilical cord blood.[1] Also, $CD4^+CD25^+$T cells present in the thymus have been reported as naïve cells that become activated and express a memory phenotype when they exit toward the periphery.[32]

Studies by Sakaguchi et al.[15,16] had already characterized the T-cell phenotype based only on the constitutive expression of the CD4 and CD25 markers, although it is known that any other $CD4^+CD25^-$ cell may, after being activated, begin to transiently express the CD25 molecule. In humans, $CD4^+$ T cells have differentiated profiles of CD25 receptor expression, with differentiated intensities detected in the medium channel of fluorescence, so that it is possible to identify, in the "gate" in $CD4^+CD25^+$ cells, a more abundant population, expressing low levels of CD25, and a lower percentage of CD4, with high intensity of expression of this receptor.[27,33] This last population, with intense expression of CD25, corresponds to the pool of this subpopulation. However, there is limitation of the CD25 receptor as a T_{reg}[20] phenotypic marker. The current strategy for isolation and characterization of $T_{regs}$is based on the recognition of this marker. CD25 also represents, in the physiology of this cell, an indispensable component for its generation and maintenance in the organism.

As previously mentioned, regarding the biomolecular approach, researchers[6,15] have demonstrated that transcription factor *Foxp3* is predominantly expressed by thymic and peripheral T_{regs}.[6,15] Naïve T cells transfected with *Foxp3* mRNA acquire characteristic of regulatory cells becoming anergic and suppressive in vitro. It was further observed that the transfected cells acquired T_{reg}-like

phenotype in relation to phenotypic expression and the production of cytokines and other T-related molecules such as CD25, CTLA-4, CD103 and GITR. Transfected cells also have the ability to suppress the proliferation of other T cells and to inhibit the development of autoimmune disease and inflammatory vessel disease in vivo.[6,34] It has also been shown that the number of T_{regs} is increased in mice transgenic to *Foxp3* and that mice KO for this gene show hyperactivation of T cells. According to previous reports,[6,15] *Foxp3* appears to be a very important gene in the development and function of $CD4^+CD25^+$ T cells, both in mice and humans.

Patients with *Foxp3* mutation have been shown to develop IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome). This condition consists of an autoimmune disorder that affects multiple organs with development of allergy and inflammatory vessel disease. These patients appear to be impaired in terms of the development of T_{regs}, thus presenting defective suppression function, which induces a state of hyperactivation of T cells that become reactive against autoantigens, commensal bacteria of the intestine or innocuous environmental antigens.[20] Most T cells expressing *Foxp3* are $CD4^+$ $CD25^{+high}$ and $CD4^+CD25^{+low}$, that is, cells with high expression of CD25 and cells with low expression of CD25 that are capable of suppressing T cell proliferation with the same intensity.

Later, Seddiki et al.[35] demonstrated that anti-CD127 monoclonal antigen is able to clearly mark the population of T_{regs}, with suppressive activity. Previous studies have reported that *Foxp3* expression did not always correlate with the expression of the CD25 molecule.[36] Liu et al.[37] found that most $CD4^+Foxp3^+$ cells were $CD25^{high}CD127^{low}$. This study demonstrated that CD25CD127 labeling was able to accurately indicate a population of suppressor T cells with a higher degree of purity, leading to the assumption that $CD4^+$ T cells could actually be significantly higher than previously thought. Thus, it is possible to distinguish clearly from a population of T cells the newly activated effector cells and memory cells, since only newly activated T cells have constitutively low expression of CD127, whereas memory cells have high expression of this marker and the traditional effector cells rapidly re-express this marker upon activation.[35-37] In addition, the use of other markers such as CTLA-4 and CD122, although also expressed under activation conditions, may aid in their characterization. In autoimmune disorders such as rheumatoid arthritis (RA), there is a lot of evidence

that the breakdown of immune tolerance mechanisms begins in the thymus with the escape of clones with self-reactive potential. In our preliminary studies, we demonstrated that the majority of the peripheral blood samples from RA patients who were evaluated had the HLA-DR marker. It is known that certain HLA-DR alleles determine both the susceptibility to the disease and its severity. These determinants, and perhaps others of a genetic nature, may be susceptible to an unidentified environmental factor. Nevertheless, progression or not to autoimmunity appears to be critically and relevantly determined in the periphery. Most often, tolerance mechanisms can control the peripheral activation of autoreactive clones that are eliminated or energized. When this control is insufficient, autoimmune disease manifests itself.

T_{reg} cells are responsible for the maintenance of "active" mechanisms of suppression and immunoregulation that work together with the other mechanisms of peripheral tolerance. Several studies have been carried out, evaluating the role of T cells in the maintenance of peripheral tolerance and the pathophysiology of autoimmune diseases. Its relevance in this process has been clearly demonstrated in murine models in which the absence or depletion of T cells triggers systemic autoimmune diseases with high titers of antinuclear antibodies as well as autologous organ-specific antibodies.[15] Important findings, such as defects in function, phenotype and frequency of immunoregulatory cells, have been reported in several human autoimmune rheumatic diseases, thus evidencing their important role in maintaining immunological tolerance and in the pathophysiological mechanisms of these diseases. The proportion of T_{reg} cells in peripheral blood was related to the observation of increased levels in peripheral blood and synovial fluid, in addition to the demonstration of suppressive activity more powerful than that observed in the peripheral ones.[38-40] In contrast, normal levels of T_{reg} in peripheral blood were also detected in some studies. This variability probably stems from differences in disease stage, therapy and certainly variations in the strategies for characterization of RAs.

In more recent research, Cao et al.[41,42] found that in approximately 95% of patients with rheumatic diseases that progress with arthritis, such as: RA, juvenile rheumatoid arthritis, ankylosing spondylitis, systemic lupus erythematosus (SLE), Behcet's disease, rheumatic polymyalgia and mixed connective tissue disease, all presenting high levels of T_{regs} in the inflamed joint, despite the clinical condition and

disease time.[41,42] These authors suggest that T_{regs}, even if numerically enhanced in inflamed synovium and with normal suppressor function, are unable to suppress secretion of proinflammatory cytokines by activated T cells or monocytes. This may occur because the suppressive action of T lymphocytes would be overcome by other lymphocytes with strong activation signals present at these sites, including Th1 and Th17 cells.[43] This fact is in accordance with the data presented by Nistala et al.,[44] who demonstrated that the balance between the populations of T_{regs} and Th17 is inversely correlated and very important in the progression of the disease.[44] Failure of the T_{regs} in RA was also suggested by Van Amelsfort et al.[45] who reported levels and suppressive activity of this increased cellular subtype in synovium of RA patients compared to the same peripheral blood population. However, inflammation persisted. Monocyte-derived cytokines, such as TNF and IL-7, as well as co-stimulatory molecules such as CD28, are possibly counteracting factors in the suppression of T_{regs} in these patients, both in synovial fluid and in peripheral blood, preventing suppression.[45]

6.2 INTERLEUKIN 10 (IL-10) IN AUTOIMMUNE AND IDIOPATHIC DISEASES

In earlier preliminary studies, the authors observed that in myasthenia gravis (MG), an autoimmune disorder, there is an increase in T_{reg} cells in the blood of individuals not treated with corticosteroids, and a decrease in $CD8^+$ T cells. The population of $CD4^+IL\text{-}10^+$ T cells obtained by Ficoll gradient separation and fluorescently labeled with monoclonal antibodies was significantly increased, with a significant reduction of clinical symptoms. In this case, IL-10 in association with CD25 appears to exert peripheral tolerance control. Studies by Falcão et al.[46] had already demonstrated a role of this cytokine in controlling exacerbated responses in infectious-parasitic diseases. In schistosomiasis mansoni, asymptomatic patients have a high IL-10 profile, together with other molecules such as HLA-DR and other co-stimulatory molecules. However, in another study, the authors reported that T_{regs} did not significantly express INF-g and inhibition of T cell proliferation was not achieved.

Studies have shown that IL-10 inhibits APC activation and is related to inflammatory control reactions in target tissues.[47]

In MG, a disease that attacks the postsynaptic portion of the neuromuscular junction and is characterized by fluctuating muscle weakness, the biological heterogeneity investigated for the first time when rabbits with acetylcholine receptors were purified to obtain antibodies against that receptor has been demonstrated. Immunized rabbits had fallen ears and palpebral ptosis (drooping eyelid) with improvement at rest and worsening with exercise, infections and emotional stress.[48] The role of these antibodies in the etiology of MG was clearly established in the 1970s, when plasmapheresis proved to be effective in the removal of antibodies and consequent functional improvement for more than 2 months.[49,50] Well-established anatomical changes were also observed, including increased neuromuscular junction size and decreased post-synaptic membrane length. Other important observations have been reported on the role of autologous antibodies in MG, since approximately 50% of patients with the disease without *Ach* anti-receptor antibodies have antibodies against a muscle membrane enzyme called muscle-specific tyrosine kinase (anti-Musk). Lavrnic et al.[48] analyzed 17 patients with this condition, observing a higher prevalence of women, predominant facial and bulbar involvement and refractoriness to anticholinesterase compounds. Because it is an autoimmune disease, other conditions of the same nature may coexist in a patient with a diagnosis of MG, and should be screened rationally, especially hypothyroidism, hyperthyroidism and thymus disease.[50] Seventy percent (70%) of patients have thymic hyperplasia and approximately 10% have thymoma – with potential for malignant behavior, which is more common in patients aged 50-70 years.

As previously mentioned, patients with clinically controlled RA had an increased T_{reg} profile; however, the authors found a differential expression of receptors on the surface of peripheral blood lymphocytes from individuals with a diagnosis of MG, with a symptom of muscle weakness, and who were treated with prednisone and azathioprine, which are immunosuppressive agents. They showed a decreased profile of T_{regs} and $CD4^{+}$IL-10, as well as increased $CD8^{+}$CTLA-4^{+} T cells.[51]

As a consequence of previous studies,[52] the authors also reported the role of these two receptors in idiopathic diseases, such as Bell's palsy. Patients with autologous

induction of IL-10, obtained through receptor purification, have been shown to have clinical improvement as well as increased T_{reg} expression. $CD4^+$ T lymphocytes were found to present increased expression after one week of induction. Paralysis affects the facial nerve (cranial nerve VII), which results in inability to control the facial muscles on the affected side. Several other conditions can also cause facial paralysis, for example, brain tumor, stroke and Lyme disease. A person may experience pain behind the ear a few hours before muscle weakness occurs. Clinical treatment includes prescribing anti-inflammatory drugs such as prednisone. Also, as in MG, immunosuppressive treatment has been used and is effective in controlling symptoms and reducing exacerbations. Pyridostigmine is reserved for refractory cases. The different dosages of glucocorticoid (daily use, alternating use or pulse therapy) do not seem to yield different efficacies.[53-55] The receptor-glucocorticoid complex enters the cell nucleus and causes some changes in the DNA that stimulate or repress the synthesis of certain tissue proteins. Prednisone is particularly effective as an immunosuppressant and alters the performance of the immune system, with a decrease in mediators for inflammation. This decrease, in certain cases, prevents the communication with other cells of the immune system that should be recruited in order to modulate the inflammatory process through the production of physiological proteins, such as interleukin 10 (IL-10). Prednisone is used in autoimmune and inflammatory diseases and, at a given moment, induces immunological immunosuppression states, precisely because it prevents the receptors fixed on the surface of the defense cells and soluble cofactors from playing their roles in cellular activation, considering that the analogous receptors of the drug can share the same ligands of IL-10, preventing its action. Prednisone is biotransformed in the liver into prednisolone by the action of the enzyme dehydrogenase 11-beta-hydroxysteroid type 1. From 1 to 3 hours after administration, the drug reaches plasma peaks. Its plasma half-life is approximately 3 hours, its biological half-life thus being 12 to 36 hours in this case.[56]

Receptors are surface proteins that bind to external signaling molecules of high affinity cells and convert this extracellular event into one or more intracellular signals that alter the behavior of the target cell. Note that the receptors for IL-10 are arranged as two-chain a-tetramers (IL-10 receptor a-chain) and two b-chains (IL-10 receptor b-chain) (Figure 2). Signaling occurs through interaction with Janus kinases. IL-10 belongs to these two receptor chains, which associate the *Jak-1* and *Tyk-2* kinases of the

Janus family. *STAT-3* is the main "downstream" signaling molecule induced by IL-10, which is produced mainly by regulatory T cells but also by macrophages and keratinocytes present in epithelial tissue.[1]*STAT-3* is expected to act to inhibit gene transcription of inflammatory and/or autoreactive receptors, forming the *STAT* complex in association with *Jak-1*and *Tyk-2*, with consequent nuclear translocation and gene activation, since mRNA of these enzymes were detected by RT-PCR, with bands of around 120KDa, with anti-janus-kinase1. The high expression of $CD4^{+}CD25^{+low}$ and $CD4^{+}IL\text{-}10^{+\ high}$ T cells found after induction of autologous IL-10 can be explained by occupying both R1 and R2 IL-10 receptors. Also, in many cases, the generation of autoreactive antibodies or T cells can also be attributed to the role played by infectious agents present in the body of the individual, such as bacteria, which lead to the generation of antibodies and T cells which, in turn, react with many different epitopes of the infectious organism. If one of these antigens is similar to an autoantigen it may result in an autoimmune responsible.[52]

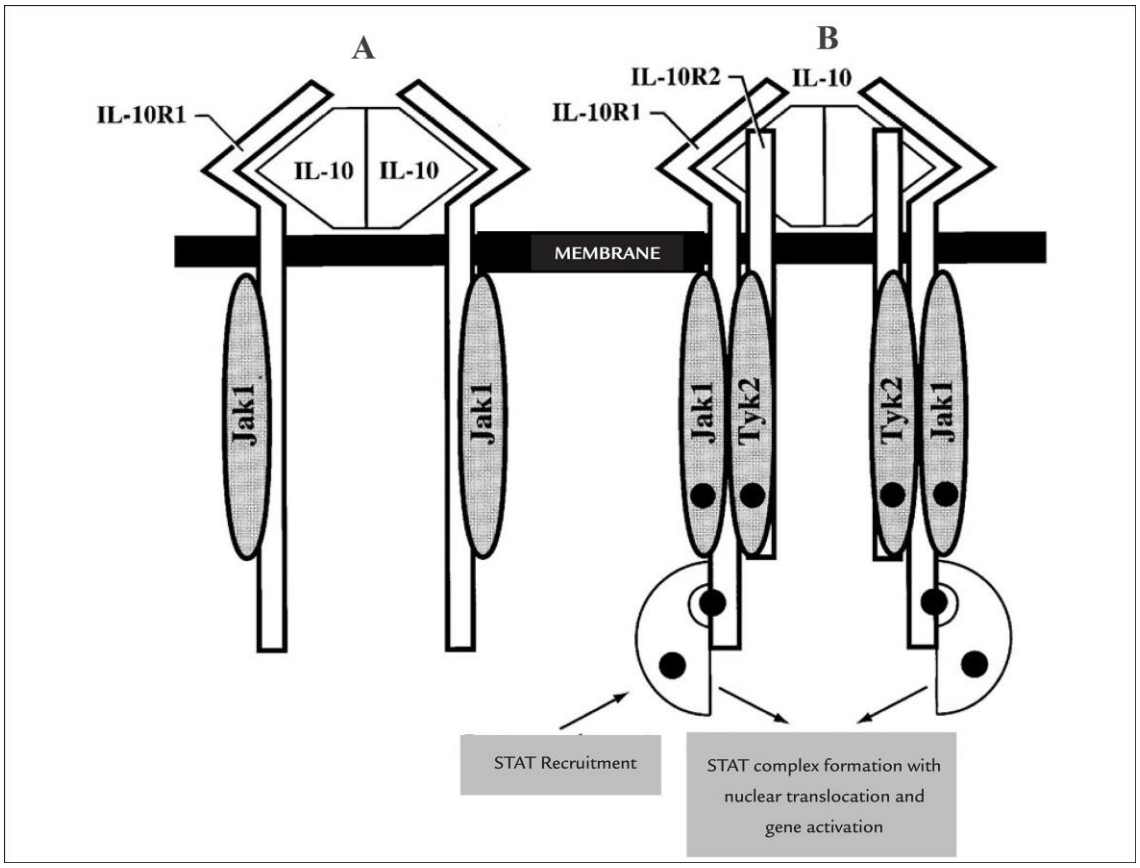

Figure 6.2- IL-10 receptor binding via *STAT-3*. A. Binding of IL-10 to IL-10R1 receptor via receptor-anchored *Jak-1* kinase. B. Binding of IL-10 to IL-10R2 receptor,

recruitment of *STAT-3* and *STAT* complex formation and gene activation by *Jak-1* and *Tyk-2*kinases. Both "outside-in" and "inside-out" signaling are associated with distinct conformational changes in the extracellular segment. These changes vary with the type and nature of the ligand and are modulated by divalent cations. (Adapted from Abbas and Lichtman,[1] 2005.)

Ex vivo monitoring of IL-10 can be obtained by analysis of human peripheral blood mononuclear cells, grown in vitro and induced by blastogenesis for the production of proteins (interleukins), through mitogenic stimulation with PHA (phytohemagglutinin). Protocols developed for in vitro and in vivo experimental phase were filed with patent application PI0206722-6, supported by experiments with animal models highly homologous to the human genome.[52] The protein fraction of IL-10 obtained by PCR and purified, free of contaminants can be analyzed by electrophoresis and quantified by UV-visible spectrometry. The procedure may become a routine.

Falcão et al.[52] demonstrated that IL-10 suspensions, ex vivo, can be applied at the inflammatory site, connective tissue and muscle. In cases of syndromes that render the synapses between first-order neurons in the periphery unfeasible, the application was close to the areas of muscle, subcutaneous or intradermal flaccidity.[52] Assuming a regular interval of 10 days between applications, the monitoring of the modulation of the inflammatory profile was performed according to Falcão et al.[52] The receptors were identified by flow cytometry, and their fluorescent histograms were prepared so that the absolute number of cell surface receptors labeled with the anti-receptor fluorescent monoclonal antibody of interest was generated, or the mRNAs for genes of the receptors were detected. As a result, they found an increase in the production of $T_{regs,}$ $CD4^{+}HLA\text{-}DR^{+}$, a decrease in $CD8^{+}CTLA\text{-}4^{+}$ and an increase in the expression of IL-10 by T_{regs} up to the fourth week, with a mean of the absolute number of receptors maintained after the sixth week. The expression of $CD8^{+}INF\text{-}g^{+}$ and $CD14^{+}INF\text{-}g^{+}$ was markedly decreased in the samples evaluated.[52]

Thus, it is conclusive that the monitoring and manipulation of proinflammatory interleukins has the potential to assist in the prognosis of anti and pro-inflammatory and degenerative changes in situ, monitoring the course of the disease.

6.3 SYNTHESIS OF TRACERS FOR IN VIVO MONITORING

Image monitoring of symptoms of autoimmune diseases, such as RA, is preferable considering that such a technique will directly contribute to the accuracy of the diagnosis and consequently the establishment of the therapeutic mode and its intensity.[57] The accurate definition of the site with a design of the inflammatory focus is relevant in the choice of therapeutic management in RA.[58] Radiological imaging, radiography, computed tomography, nuclear magnetic resonance or ultrasound may favor an analysis of the deleterious effects on the anatomical structures in the peripheral joints.[59] However, such images do not aid in the early analysis of RA. Scintigraphy, on the other hand, may promote an early diagnosis of inflammatory processes by monitoring the early stages of inflammation. Thus, radioisotope scintigraphy is expected to contribute to the diagnosis of RA by monitoring functional and physiological changes at the inflamed site before anatomical structural changes consequent to RA can become apparent.[60]

Positron-emitting fluoride-18-labeled deoxy-glucose (FDG) is a radiopharmaceutical used in positron emission (PET) scintigraphy. The compound accumulates in the inflammatory site, given the high local metabolism. The high supply of leukocytes in the inflamed site leads to increased glucose consumption.[61] However, due to the high cost of production of this 110-minute half-life radiopharmaceutical, together with the cost of PET imaging, it is currently impracticable to perform systematic clinical studies of RA using this technique. Cost reduction or new methods and radiopharmaceuticals should be produced to enable scintigraphy of RA.[61] The use of radiolabeled ex-vivo leukocytes is attractive; however, they involve difficult management with high control of sterility and apyrogenicity.[62] Although leukocyte scintigraphy radiolabeled with ^{111}In and ^{99m}Tc is a gold standard for the diagnosis of inflammation, the process of marking autologous leukocytes with ^{99m}Tc-HMPAO demands manipulation of blood samples in aseptic facilities with the reintroduction of these samples into the patient.[63]Obviously, there is the inherent risk of contamination, during manipulation of PBMC cells and isolation and labeling of leukocytes.[63]

A recent patent PI0904754-9, developed by the research group coordinated by the author, has shown that Tc-99m-labeled thio-sugar analogues of glucose are efficient

in detecting inflammations.[64] Previous synthesis studies had been successfully performed using 5-thio-D-glucose; however, due to cost issues, there was a need to replace the thio-sugar molecule.[65] The importance of thio-sugars in inflammations was demonstrated in the temporomandibular joint (TMJ) of rats.[63] The patent involves 5-thio-glucose and 1-beta-thio-D-glucose labeled with Tc-99m.[64] The results show significant differences in the uptake of ^{99m}Tc-1-TG in the inflamed TM joint compared to the control, with high renal excretion. Tc-99m-labeled glucose analogs may become radiopharmaceuticals important for detection in the monitoring of inflammations such as AR due to the low cost and high technological feasibility. However, despite the murine investigations, there is still a need for clinical investigations demonstrating its efficiency in the early detection of RA and the degree of disease involvement in humans before and after immunological treatment.

Research on the synthesis and characterization of sugars with heavy metals has advanced. Recently, Dalmazio and Campos[66] demonstrated by mass spectrometry the viability of direct labeling of sugar polymolecules with Sm, Gd, B, Li, Tc, Sm, Ho, Eu, and other elements. These metal-sugar complexes make it possible to define several tracers for different modalities of medical imaging tests. These studies lack in vivo experimentation, but already offer a promising perspective in the monitoring of autoimmune diseases.

IL-1 and IL-6 interleukins play a crucial role in RA and osteoarthritis in the early processes of cartilage breakdown and destruction.[65] A significant increase of IL-6 in patients with osteoarthritis was identified by Kaneyama et al.[67]In 2014, in turn, Sukedai et al.[68] report the relation between TNF-a and cartilage degeneration. These authors show that IL-8 is closely involved with the acute phase of the inflammatory process. Thus, interleukins, such as IL-1, IL-6, IL-8, are proteins with which in vivo monitoring may lead to differential diagnosis of RAs.

Radiolabeled sugars serve the monitoring of inflammation induced by autoimmune diseases; however, they are not specific. It is worth saying that the interleukins themselves have high potential for radiolabeling. Rennen et al.[61,69] performed the labeling of IL-8 with Tc-99m making it possible to diagnose inflammation through radiolabeled interleukins.[69] Thus, we conclude that inflammatory cytokines are potential markers to aid in the diagnosis and prognosis of anti- and pro-

inflammatory and degenerative changes in situ, monitoring the course of the disease. Radiolabeled cytokines, together with high metabolism labeling radiopharmaceuticals, represent a promising class of compounds for the evaluation of autoimmune diseases since these proteins play an important role in inducing and maintaining the disease process.

6.4 CONCLUSION

The present review addressed cellular markers whose analysis and modulation may be useful in the treatment of autoimmune and idiopathic diseases, as well as in the prognostic monitoring of diseases. It has been noted that the ex-vivo monitoring and manipulation of interleukin IL-10 is relevant for treatment, and that thio-sugars, monossacharides, polysaccharides and radiolabeled interleukins are tools for in vivo monitoring of autoimmune and idiopathic diseases. Future consolidation of scintigraphic methods can help monitor the progression of such diseases. Advances in research on modulation and generation of radioactive drugs involving cell markers for diagnosis and therapy may bring benefits to patients with autoimmune diseases.

Study conducted at Departament of Nuclear Engineering, Program of Nuclear Science and Techniques, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, MG, Brazil

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CAPITULO 7

TOWARD COADJUVANT ONCOLOGIC THERAPIES MODULATING SIGNALING PATWHWAYS – A SHORT REVIEW

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7.1 Introduction

Carcinogenesis is a process where the physiological function of live cells is altered, resulting in the abnormal and uncontrollable growth of a given organ or tissue [1] The concept that cancer originates from uncontrolled cell division mechanisms is relatively recent. It is known that cell division is controlled by a network of signals acting in synergy, determining the exact moment of division, its frequency, and how the eventual errors can be repaired.[2] Mutations in one or more segments of such a network may trigger abnormal neoplastic growth. Despite cancer is coin a genetic

disease, it is unlikely that a single genetic change or a single agent could explain the onset of cancer. Studies on cancer are characterized by enormous efforts as well as by considerable public and private expenditures. Studies regarding its causes have mostly been of an epidemiological character, with the support of experimental techniques of cellular and molecular biology [2,3].

Holistic thesis toward coadjuvant treatments in oncology

The insight that the tumoral growing strongly responds to a hormonal and immune signaling pathways, triggered by diverse tissues of the human body, modulated by natural chemical compounds, superimpose to the restrict view that the cancer is driving by a DNA alteration in a genetic cell-level phenomenon. A holistic understanding is proposed, rather than an exclusively cell-intrinsic mutation, toward coadjuvant treatments in oncology.

In opposing such thesis, the current cancer treatment, including surgery for tumor resection, radiation therapy of the tumor bed, and chemotherapy targeting antimitotic drugs, are linked to suppression of mutagenic cells with carcinogenic genes; supporting the restrict view of extirpation of carcinogenic clones.

7.2 Ionizing Radiation in Cancer

Radiation as an integral part of the cancer treatment

Concerning the therapy in cancer, radiotherapy is an integral part of the primary conservative treatment of various types of cancer and has been a good option for efficient treatment. Considering as an example, high invasive potential breast carcinoma [4,5], modern radiotherapy aims to achieve a favorable therapeutic index, aiming at a

better local control of the tumor, leading the malignant cells to lose their clonogenicity, preserving the functions of healthy tissues.

Intrinsic cellular radioresistence and radiosensibility in radiation therapy

It is important to mention that the clinical response to radiotherapy is related to target cells radio sensitivity and resistance, which are associated with the clonogenicity activity of the cell lines in the study and their sensitivity to radiation [6,7]. The more undifferentiated and proliferate the tissue, the most sensitive it is to radiation, while on the other extreme, the more differentiated and stable the tissue, the more resistant it is. In such a context, erythroblasts and spermatogonia are more sensitive while muscle and nerve cells are more resistant. On the other hand, lymphocytes are non-clonogenicity differentiated cells, being among the most radiosensitive cells in the body, because of their susceptibility to radiation-induced apoptosis [7]. In such context, it is important to evaluate the variation of irradiated cancer cells clonogenicity response in radiosensitive and radio resistant *in vitro* model, as a function of receiving radiation dose and dose rate, in the dose domain of radiation therapy. Thus, it is possible to verify the modulation of the effects of ionizing radiation assisted by the dose, dose rate, radioprotectors or radiosensitizer concentrations, or other coadjuvant factors, useful in radiotherapy.

ROS in radiation therapy

The radiation therapy (RT) induces the malignant cells to lose its clonogenicity, probably by the inhibition of the pathways of cellular signaling, and, in contrast, preserves as maximum as possible the functions of healthy tissue. RT presents great efficiency in the control of the tumors, applying external radiation provided by X-ray beams of continuous spectra from linear accelerators. The primary gamma-radiation interacts with the atoms of the tissue and produces secondary scattering electrons that ionize the medium and create primarily free-radicals, such as OH, H., e_{aq}-. Such primary radicals interact with dissolved oxygen and the free-radicals themselves, producing

secondary radicals, such as the radicals O_2^-, HO_2, H_2O_2. Those free radicals spread out by diffusion in the cell and can provoke the single (SSB) or double (DSB) rupture of the DNA chains. The oxidative interactions of the free radicals can lead the cell to death through various mechanisms, through the inactivation of vital cellular systems that disable the clonogenic reproduction [9]. However, studies have demonstrated that the irradiation of breast tumor with low doses and dose rate can induce radio resistance. [10,11]. Indeed, not only dose, but the dose rate, as already demonstrated by Falcão and cols, 2015, possess clinical relevance, being able to reduce the tumor control in breast cancer in cobalt therapy [11]. Therefore, it is worth mention that the success of the tumor control depends not only on the best choice of the physical parameters of the modern radiation therapy, but also on the general health status and the response of the immune system of the patient, including the intrinsic capacity to control the number of trigger signals started by the tumor, responsible for the maintenance of its clonogenicity.

7.3 Hormones in Cancer

Hormone role in treating cancer

In this context, it is worth noting that the cofactors secreted at the cellular level and / or induced by the hypothalamus in response to specific treatment are fundamental for tumor control, as they may be indirectly or directly involved in triggering clonogenesis and a possible recurrence. For example, in breast cancer, hormone therapy is only used in cases in which cells are hold a positive receptor since such tumor cells grow induced by hormonal stimulation. Therefore, only those that have estrogen and / or progesterone receptors on their surface are receptive to the treatment. These receptors act as estrogen ligand, allowing the cell internalization and functional modulation. [12]

The pathway of estrogen production begins in the hypothalamus, which "sends" signaling to the pituitary gland for synthesis and production of various hormones, including gonadotrophic hormones FSH (Follicle Stimulating Hormone) and LH (Luteinizing Hormone). These hormones will act on the female sex gland - ovaries - resulting in the synthesis of estrogens. Estrogen can still be produced to a lesser extent by another gland, the adrenal gland. Blockade of this production pathway, in its different stages, is the main target of hormone therapy for breast carcinoma.

Adipose tissue as a endrocrinum organ driving a tumoral response

Fat tissue produces diverse derived hormones, such as such as leptin, estrogen, resistin, adiponectin, plasminogen activator inhibitor-1, estradiol, and cytokine (especially TNFα, IL-6) namely adipokines that is signaling proteins [11a].

Woman adipose tissue (fat cells) is an estrogen producing site. When of childbearing age (menstrual cycles), the production of female hormones is primarily synthesized in the ovary, but after menopause the adrenal gland is responsible for this function, producing hormones that will later "convert" into estrogen in the adipose tissue. Therefore, obesity, overweight, higher waist circumference are linked with a higher hormone production in menopause, and consequently, higher risk of primary development or recurrence of breast cancer. Weight reduction is thus indirectly a hormone therapy for cancer control. Currently, the drugs used in an attempt to reduce the action of estrogens on cancer cells act primarily in two predominant ways, suppressing the plasma concentration of female hormones in the body or blocking the signaling of the hormones into the cells [12].

Hormones driving ovarian cancer

Ovarian cancer is the sixth most common cancer worldwide among women in developed countries and the most lethal of all gynecologic malignancies [13] About 90% of primary malignant ovarian tumors are epithelial carcinomas and are further classified as serous, endometrioid, clear cell, mucinous, transitional, mixed cell, or undifferentiated based on cell morphology [14]. Recent technological advances have shed light on both the cellular and the molecular biology of ovarian cancer such that it is now widely believed that `ovarian cancer' is a general term for a group of molecularly and etiologically distinct diseases that share an anatomical location [14]. In particular, the diverse histological subtypes of carcinomas are believed to originated by histological-similar epithelial cells derived from different tissues. For example, high-grade serous carcinomas are believed to arise from the ovarian surface epithelium and/or the distal fallopian tube [15], whereas endometrioid and clear cell carcinomas are believed to arise from endometriotic lesions [16]. In contrast, most mucinous tumors are believed to be metastases to the ovary from the gastrointestinal tract, including the colon, appendix, and stomach [17, 18, 19].

Despite the differences in the putative tissues of origin of epithelial ovarian cancers (EOC), the presence of sex steroid hormone receptors in many of these tissues that gives origin for ovarian cancer [20, 21, 22, 23], as well as in many malignant epithelial ovarian tumors [24, 25], suggests a potential role for hormones in the origin and promotion of these diseases. However, studies in the signaling pathway mechanism are lacking, and models to study hormone response *in vitro* and *in vivo* are very limited.

7.4 Signaling pathways from CNS and imune system concerning cancer

CNS Network signaling pathways in regulating immune system toward tumor control

The understanding the physiological mechanisms involved in regulating the immune and nervous systems has early been the focus in biomedical research. Previously attention to understanding these processes was in an isolated and fragmented manner, given way a more synthetic and integrated view of the complex signaling pathways amoung the organs, tissues, and cells [44]. In fact, it has come to light that the immune system is part of a much larger control network.

Several CNS stimuli are now known to be part of the signal pathways that modulate an immune response. The endocrine system - and in particular the Hypothalamus-Pituitary Axis. adrenal (HPA) - is responsible for several of the links between the Central Nervous System (CNS) and Immune Systems [45]. This response also includes endorphins, thyrotropin, prostaglandins, growth hormone and, especially, the sympathetic autonomic nervous system (SNAS). The activation of the HPA axis and the consequent production of glucocorticoids during stress are one of the main mechanisms responsible for the alterations of the immune response found during this process. Glucocorticoids are known to inhibit the transcription of numerous cytokines such as interleukin 1 (IL-1), IL-13, IL-5, IL-6, IL8, tumor necrosis factor (TNF) and Stimulating Factor Colony (GM-CSF) [46]. They also inhibit eosinophil and neutrophil migration, including chemotaxis [47]. Perhaps this is why it has been found, in clinical studies, the relationship between stress and immunological parameters in which stress was positively correlated with leukocytosis, decreased NK cell count, increased CD4+ / CD8+ ratio, and decreased T and NK activity [48]. It worth to mentioning that one of the most relevant mechanisms of stress immune modulation via HPA axis activation is due to changes in the so-called TH1 / TH2 balance [49].

In this context, immune responses are regulated by antigen presenting cells (monocytes / macrophages, dendritic cells and other phagocytes) - which are components of the so-called innate immune response - and also by lymphocytes of the subclasses TH1 and TH2, which make up the acquired calling response. Basically, what differentiates these two lymphocyte populations is the cytokine profile present on the signaling pathway that they command. Thus, the subpopulation of TH2 lymphocytes secretes cytokines such as IL-12, Interferon-γ (IFN-γ) and TNF-α; These cytokines act as promoters of cellular immune activity. Already the subpopulation of TH2 lymphocytes releases, among others, IL-4, IL-9, IL-10 and IL-13, which stimulate humoral immune activity [50, 51]. Thus, when produced, IL-12 and TNF-α increase the innate immune response or TH1 and inhibit humoral or TH2 responses [51].

Interleukins IL-4 and IL-10 produce the opposite effect, ie displace TH1 balance / TH2 to the TH2 standard. Considering this balance, stress - via glucocorticoid secretion - favors TH2.[52] type responses. Glucocorticoids, acting on monocytes, macrophages and dendritic cells inhibit IL-12, TNF-α and INF-γ production, directing lymphocyte differentiation to the TH2 profile [53]. Thus, there is a decrease in cellular immune response and an increase in humoral response; the susceptibility to allergies and antibody-mediated autoimmune diseases increases in the stressed patient.[50]

Given the theory and facts that support the correlation between the CNS and the IS, it is challenging that the behavior of the IS, in the face of cancer therapies and treatment success or possible recurrences may be directly connected with the network of signals triggered by the CNS, including direct binding of SI to tumor sites.

Signaling of Metalloproteinases and TGF-beta in cancer progression induced by UV radiation

Falcão et al, 2014 investigated the profile of metalloproteinases and TGF-beta in epidermal cells of patients undergoing UV radiation. which may be associated with a poor cancer prognosis. This study showed that UV radiation emitted by the solar simulator was able to stimulate extracellular matrix cells in vitro culture for the production of TGF-beta, MMP-2 and MMP-9 expressions and their mRNAs. Since these MMPs and TGF are related to cancer evolution and its pathogenesis, these findings confirm that UV radiation may contribute to the prognosis of such diseases based on MMP and TGF-beta secretion [44].

7.5 The role of phytotherapy drugs in signaling pathways

The role of Phytotherapy coadjuvant in cancer

Recently, studies have suggested a probable action of some natural active substances in cancer, holding a perspective of a cancer adjuvant treatment in complementation to the traditional interventions. [43]

Endogenous enzymes (glutathione, superoxide dismutase, catalase) or hormones (melatonin), vitamins (C and E vitamins), carotenoids and phytochemicals (flavonoids and curmin) were already addressed as radioprotectors or radiosensitizers coadjuvant to RT .

The broad spectrum of Propolis as phytotherapic

The Propolis is a resinous substance collected by bees from different plant parts with antioxidant properties. It is known to contain a variety of chemical compounds as steroids phenolic acids, esters of phenolic acids, flavonoids and terpenoids, such as CAPE and Artepillin C [26, 27, 28, 29]. Propolis possesses biological active substances, including antibacterial, antiviral, antioxidant, anti-carcinogenic, and anti-inflammatory effects [30].

Propolis in conjunction of RT provides a measurable protection against DNA damage caused by ionizing radiation in leukocytes of patients during radiotherapy treatment [31]. In addition, the immune activity provided by the use of Propolis and related compounds increase hematopoietic regeneration and survival after induction by radiation [32]. Studies on the effect of Propolis and its property, extensively in vitro and *in vivo,* have been brought attention on the literature [33, 34].

Signaling pathway modulated by Propolis in Radiation Therapy

The present authors demonstrated modulations of TGF-β and IL-6, expressed by the radio resistant breast adenocarcinoma cells after a low dose of radiation of low LET, with significantly increasing in comparison to the unirradiated control. The dose of 2 Gy induced the reduction of TGF and IL-6 after 24 h; and past 72 h, the cellular environment had recovered the control levels. This fact represents an unfavorable situation, suggesting that the dose of 2 Gy (considered a conventional daily fractional dose in RT) is not efficient to keep the reduction of tumoral clonogenesis *in vitro* in the irradiated conditions. These data are corroborated by Divella et al, 2013, that demonstrated the clinical impact of the serum TGF-beta and CXCL1 chemokines, as predictive factors for clonogenesis and also for the cell escape to the circulatory system, associating it to low prognostic of this illness [35]. A high level of TGF-β seems to favor a more aggressive clonogenic phenotype promoting the growth of the tumor, extending the resistance to apoptosis, increasing the mobility of the cancerous cells and eventually metastases [36, 41].

It is interesting to observe that, in some types of breast tumors, the in situ cellular surrounding tissue, that compose the tumoral microenvironment, seems to be involved in the maintenance of a high level of TGF-β, through specific processes of signaling [37, 42]. In MDA-MB-231 culture, TGF-β was detected prominently at earliest 24 h in the supernatant and later observed a decline that can be associated to performances of some transcription factors, in the presence of Propolis extract.

A high level of TGF-β seems to favor a more aggressive clonogenic phenotype promoting the growth of the tumor, extending the resistance to apoptosis, increasing the mobility of the cancerous cells and eventually metastases [36]. It is interesting to observe that, in some types of breast tumors, the in situ cellular surrounding tissue that compose the tumoral microenvironment, seem to be involved in the maintenance of a high level of TGF-β, through some specific processes of signaling [36]. In MDA-MB-231 culture, TGF-β was detected prominently at earliest 24 h in the supernatant and later observed a decline that can be associated to specific performances of some transcription factors. Considering that NF-κB and STAT3 pathways are chemotherapy targets in various types of breast tumors, it is possible to infer that they can mediate the regulation of TGF-β [37]. Hendrayane et al., 2014, had demonstrated an association between TGF-β1 and IL-6 since the TGF-β1 can mediate the negative regulation of IL-6. This observation was possible found through experiments with co-culture of isolated fibroblasts removed from histological sections of healthy breast tissue, surrounding the tumor. Therefore, it is worth mentioning the relevance of reproducing the most as possible the tumoral microenvironment in the in vitro assays.

On the other hand, the modulation of the tumor targets, as cytokines or chemokines, to extend the control of the oncologic illness and to reduce the recurrence [37]. The modulation of tumor targets may regulate the intensity and duration of some clinical specific responses, as well as may alter the processes of cellular conscription in favor of cancer [37].

The use of natural substances becomes attractive, as a coadjuvant therapy when they hold the potential to modulate the cytokines in the tumor environment. In special, it is worth investigating the natural substances that can act and modulate the tumoral targets, with the potential coadjuvant role in the radiation therapy of radio resistant tumors, as the MDA-MB-231 cellular case. The aqueous Propolis extract may have held this role in the assays submitted to 2 Gy of irradiation from Co-60.

The TGF-β and IL-6 in the supernatant of the MDAMB231 cultures were significantly affected in the group that received 10 % of the extract, suggesting that the Propolis can be associated with the inhibition of these two pro-clonogenic co-factors in the in vitro assays.

Studies on the regulation of the clonogenesis of MDA-MB-231 cells have boarded the role of the IL-6 in the modulation of p16, p21, and p53 (proteins suppressors of oncogenes) and in the cellular differentiation of fibroblasts to myofibroblasts. It is well known that the anti-IL-6 monoclonal antibodies have influenced the IL-6 expression positivity [38].

On the other hand, the addition of recombinant IL-6r to the cultures of the radioresistant MDA-MB-231 lineage was capable to reduce the levels of expression of mRNA for these three tumor suppressor proteins, suggesting the regulation of the paracrine effect of MDA-MB-231 on the expression of oncogenes [39]. Studies showed that IL-6 actives the JAK/STAT3 pathway and this route is capable to suppress p21 and p53 in fibroblasts in the stroma in the early 24 h in culture. Moreover, the molecular mechanism of suppression of p16, p21, and p53, dependent on STAT3, seems to involve another protein, the AUF1, whose modulation of these suppressor genes occurs post-transcription [40, 41, 42, 43].

As mentioned, *in vitro* studies of highly malignant and radio resistant cell culture have addressed the role of natural substances in pro-tumor receptor signaling

after a low radiation dose (corresponding to fractional doses in RT). Possible modulation in the expression of solid pro-tumor cytokines, especially TGF-beta and IL-6 has been demonstrated in the presence of the aqueous extract of *Tetragona clavipes* Propolis. Thus, it is pertinent to evaluate the effect of propolis extract on the possible transcriptional pathways of tumor suppressor genes by manipulating recombinant cytokines and monoclonal anti-IL-6 and anti-TGF-β antibodies in culture. The expectation of elucidating not only the adequate radioresistance of the MDA-MB-231 strain, but also the role of *Tetragona clavipes* propolis in the negative regulation of tumor cytokines, supporting a future natural co-adjuvant treatment for RT in breast cancer.

There is a prospect that the supporting potential of *Tetragona clavipes* propolis may extend to other compartments of the human organism. As the subject has already been reported in studies with radio resistant cell models, it is possible that propolis extract also acts on extracellular matrix cells, considering co-factors secreted by tumor-susceptible epidermal cells.

A probable mechanism of Propolis action on modulation of pro-tumor cytokine production should be questioned and the mechanism may also involve a nuclear microenvironment.

7.6 Final remarks

The signaling pathway of diverse cytokines, modulated by natural substances, may alter the microenvironment of the tumor contributing to the suppression of the tumor growing and its development, migration and metastasis.

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CAPÍTULO 8

8.1 CONCLUSÃO GERAL

A investigação do papel de produtos naturais, como a própolis, em estudos *in vitro* e *in vivo*, quando finalizados, poderão suportar os tratamentos radioterápicos atuais, e provavelmente diminuir o tempo de tratamento, elevando a sua eficiência, uma vez que o efeito radioprotetor da própolis parece contribuir para a eficiência do sistema imunológico em resposta ao fracionamento de doses de radiação prescritas nos planejamentos radioterápicos em diversos tipos de tumores.

Os achados que demonstram o papel interleucinas na modulação da clonogênese tumoral consubstanciaram a utilização de substâncias naturais como a própolis em terapias combinadas à Radioterapia (RT), vista que a utilização da mesma apresenta um apelo anti tumoral. Os estudos *in vitro,* sem o uso da própolis sugerem que tanto a viabilidade celular, quanto a produção de interleucinas pró-tumorais parecem sofrer alterações significativas na indução do seu potencial agressivo, observado em culturas de células de adenocarcinoma de mama da série MD. Ademais, existe a especulação de que haja desbalanço na produção de interleucina (IL-10), em detrimento da alta clonogenicidade das linhagens tumorais agressivas e radio resistentes, ou mesmo essa interleucina não estar sendo produzida suficientemente pelas células do sistema imune, já que o mesmo poderia estar sendo bastante recrutado para combater a própria imunossupressão desencadeada como consequência da RT, acelerando a hematopoiese com células ainda não habilitadas.

Vale ressaltar que as terapias atuais do câncer e sua associação tem mostrado sua relevância para a investigação do papel do extrato de própolis *Tetragona clavipes* na

inibição de interleucinas pró-tumorais produzidas pela linhagem de adenocarcinoma de mama MDAMB231 irradiadas com Co-60 de baixa dose, após a caracterização bioquímica do extrato aquoso da própolis da espécie *Tetragona clavipes*

Os nossos resultados demonstraram modulações de TGF-β e IL-6, expressas pelas células de adenocarcinoma mamário resistentes a radiação após uma baixa dose de radiação de baixo LET, com aumento significativo em relação ao controle não irradiado, sugerindo uma situação desfavorável, a dose de 2 Gy (considerada uma dose fracionária diária convencional em RT) não parece ser eficiente para manter a redução da clonogênese tumoral in *vitro* nas condições irradiadas. Um alto nível de TGF-β parece favorecer um fenótipo clonogênico mais agressivo promovendo o crescimento do tumor, ampliando a resistência à apoptose, aumentando a mobilidade das células cancerosas, o que poderia levar eventualmente a metástases.

Como perspectivas futuras, seria pertinente avaliar-se o efeito do extrato de própolis sobre as possíveis vias de transcrição de genes supressores de tumor por meio da manipulação de citocinas recombinantes e anticorpos monoclonais anti-IL-6 e anti-TGF-β em estudos *in vitro.*

Vale considerar que, estudos de proteômica deveriam ser os próximos passos para se aprofundar nos processos biológicos que ocorrem pós-irradiação, principalmente nas linhagens radio resistentes, uma vez que algumas proteínas específicas podem exercer a função de recomposição do genoma, sem quebra de ciclo. Para isso, existe atualmente a disponibilidade de ferramentas que podem determinar a localização e distribuição dinâmica de proteínas entre as organelas, que parece ser crucial para o entendimento da regulação dos processos celulares.

Considerando que o nível de complexidade do proteoma humano se estende muito além do número de produtos gênicos expressos pelo genoma em uma célula e os estudos de localização de proteínas forneceram novos modelos para ligar mutações a certos distúrbios e distúrbios na localização subcelular de doenças humanas, a investigação da localização subcelular de proteínas intracelulares é relevante para entender como sua dinâmica e função podem estar ligadas às mutações induzidas pela radiação. Basicamente, a localização de proteínas por marcação de isótopos (LOPIT- Localization of Proteins by Isotope Tagging) é um método bem estabelecido nesta área. Ele atinge a separação de alta resolução de compartimentos subcelulares de forma eficiente. A localização de proteínas por marcação isotópica após ultracentrifugação diferencial

(LOPIT-DC) irá facilitar a identificação de localizações de isoformas específicas para proteínas em estruturas suborganelares, complexos de proteínas e vias de sinalização e fornecerá um mapa de alta resolução da localização subcelular de proteínas.

A expectativa é a de elucidar não somente a radio resistência das cepas da série MD MDA-MB-231), cujo impacto no prognóstico é sombrio, mas também o papel que própolis *Tetragona clavipes* na regulação negativa de alvos tumorais (proteínas tumorais), subsidiando um futuro tratamento coadjuvante natural eficiente para RT no câncer de mama.