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Uncaria tomentosa: POSSÍVEL USO COMO ADJUVANTE NO TRATAMENTO DO CÂNCER CÓLON RETAL

TESE DE DOUTORADO

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Santa Maria, RS, Brasil

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Uncaria tomentosa: POSSÍVEL USO COMO ADJUVANTE NO TRATAMENTO DO CÂNCER CÓLON RETAL

Iria Luiza Gomes Farias

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de

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Orientador: Profa. Dra. Maria Rosa Chitolina Schetinger

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Uncaria tomentosa: POSSÍVEL USO COMO ADJUVANTE NO TRATAMENTO DO CÂNCER CÓLON RETAL

elaborada por
Iria Luiza Gomes Farias

como requisito parcial para obtenção do grau de
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Santa Maria, 23 de setembro de 2011.

DEDICATÓRIA

À Júlia.

Que bom que o tempo dá voltas,

e que esses ciclos da vida permitem encontros como esse:

Trabalhar com minha filha e encontrar nela uma colega competente!

Que bom!

AGRADECIMENTOS

Aos pacientes e familiares que confiaram em nosso trabalho e aceitaram participar da pesquisa;

A todos os colegas-amigos, binômio fundamental para o êxito de todo trabalho, que de perto ou de longe colaboraram direta ou indiretamente com nossas pesquisas;

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Ao CNPq e CAPES pelos recursos financeiros, ao Herbarium Laboratório Botânico pela cooperação técnica, doação dos comprimidos de Unha de Gato® e do extrato seco de *Uncaria tomentosa;*

Muito obrigada!

EPÍGRAFE

“**Reactive oxygen species in cancer cells:**
Live by the sword, die by the sword.”
Paul T. Schumacker, Cancer Cell, 2006

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica

Universidade Federal de Santa Maria

Uncaria tomentosa: POSSÍVEL USO COMO ADJUVANTE NO TRATAMENTO DO CÂNCER CÓLON RETAL

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Os relatos da utilização da unha de gato (*Uncaria tomentosa)* em pacientes com câncer, em conjunto com terapias tradicionais, como quimioterapia e radioterapia, referem atenuação sobre os efeitos adversos a essas terapias (como perda de cabelo, perda de peso, náuseas, infecções secundárias e problemas de pele). Pesquisas demonstraram que a unha de gato pode ajudar na restauração do DNA celular, prevenir os danos celulares causados por quimioterápicos, atuar como imunomodulador e antioxidante. Estudos também demonstram que a unha de gato pode ter um efeito mieloestimulante direto. A dose terapêutica dos quimioterápicos é frequentemente restrita pelos efeitos tóxicos, sendo a neutropenia a forma mais observada, aumentando o risco de infecções. As intervenções farmacológicas capazes de reduzir ou prevenir os efeitos adversos podem ter um impacto substancial sobre o tratamento do câncer. Neste contexto situam-se os objetivos deste trabalho: estudar a fisiopatologia do câncer colón retal e avaliar o efeito da *Uncaria tomentosa*, utilizada como adjuvante no tratamento do câncer colón retal, nos parâmetros hematológicos, imunológicos, estresse oxidativo e danos ao DNA. Os resultados (i) corroboram estudos prévios que indicam a influencia do sistema imune na evolução do câncer cólon retal e revelaram o risco relativo de pior resposta clínica do grupo das mulheres, por apresentarem menores valores de células T $CD8^+$ e baixos níveis de hemoglobina; (ii) demonstram que o processo adaptativo das células tumorais ao metabolismo glicolítico, em consequência da oxigenação deficiente, resulta em maior peroxidação lipídica, mensurada pelo conteúdo de TBARS e que este parâmetro é um indicador precoce da não resposta clínica; (iii) comprovam que a *Uncaria tomentosa* possui propriedade de estímulo a proliferação mielóide, semelhante ao fator estimulante de colônias de granulócitos, sendo eficiente na recuperação da neutropenia induzida por quimioterapia, em modelo animal; (iiii) mas, na dose de 300 mg de extrato seco/dia, a *Uncaria tomentosa* não é efetiva em alterar a resposta imunológica; em melhorar os níveis do estresse oxidativo e reduzir os danos ao DNA; em minimizar os efeitos colaterais da quimioterapia dos pacientes com câncer cólon retal. A *Uncaria tomentosa* é eficiente em reduzir a neutropenia causada por quimioterapia, por estimular a proliferação dos precursores mielóides, sendo necessários outros estudos para determinar em quais esquemas terapêuticos o seu uso pode ser efetivo.

Palavras chave: Câncer. Estresse oxidativo. Mieloproliferação. Neutropenia. *Uncaria tomentosa.*

ABSTRACT

Doctoral Thesis

Biologic Sciences Graduate Program: Toxicological Biochemistry
Universidade Federal de Santa Maria

Uncaria tomentosa: POSSIBLE USE AS AN ADJUVANT IN THE TREATMENT OF COLORECTAL CANCER

AUTHOR: IRIA LUIZA GOMES FARIAS

ADVISER: MARIA ROSA CHITOLINA SCHETINGER

Date and Place of Presentation: Santa Maria, September 23th 2011.

Reports of the use of cat's claw (*Uncaria tomentosa*) in patients with cancer, in conjunction with traditional therapies such as chemotherapy and radiotherapy, show decrease in adverse effects to these therapies (such as hair loss, weight loss, nausea, secondary infections, and skin problems). Subsequent research showed that cat's claw may help in the restoration of cellular DNA, preventing cell damage caused by chemotherapy; it also acts as an immunomodulator and antioxidant. Studies also show that cat's claw may have a direct effect in stimulating myeloid. The therapeutic dose of chemotherapy is often limited by toxicities, neutropenia being the most observed at high risk of infection. Pharmacological interventions able to reduce or prevent adverse effects may have a substantial impact on the treatment of cancer. In this context, it follows the objectives of this research: to study the pathophysiology of colorectal cancer and evaluate the effect of *Uncaria tomentosa*, used as an adjunct in the treatment of colorectal cancer in hematological, immunological, oxidative stress and DNA damage. The results (i) confirm previous studies that indicate the influence of the immune system in the evolution of colorectal cancer and showed the relative risk of poor clinical response of the group of women, since they have lower levels of T CD8+ cells and low hemoglobin levels; (ii) it showed that the adaptive process of tumor cells to glycolytic metabolism as a result of poor oxygenation, resulting in increased lipid peroxidation measured by TBARS content and that this parameter is not an early indicator of clinical response, (iii) it proves that *Uncaria tomentosa* has the property of stimulating myeloid proliferation, similar to colony stimulating factor of granulocytes, being effective in recovery from neutropenia induced by chemotherapy in animal models, (iv), although, in the dose of 300 mg of dry extract per day, *Uncaria tomentosa* was not effective in altering the immune response, in improving the levels of oxidative stress and in reducing DNA damage, and in minimizing the side effects of chemotherapy of colorectal cancer patients. The *Uncaria tomentosa* is efficient in reducing neutropenia caused by chemotherapy, by stimulate the proliferation of myeloid precursors, further studies are needed to determine in which treatment regimens its use may be effective.

Keywords: Cancer. Oxidative stress. Myeloid proliferation. Neutropenia. *Uncaria tomentosa*.

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LISTA DE ABREVIATURAS E SIGLAS

AOT	Alcalóides Oxindólicos Tetracíclicos
ADA	Adenosina Deaminase
CD39	NTPDase (Ecto-Apirase)
CD73	5'-Nucleotidase
CEA	Antígeno Carcinoembrionário
CFU_GM	Unidades Formadoras de Colonias de Granulócitos e Macrófagos
CSFs	Fatores Estimuladores de Colônia
EROS	Espécies Reativas de Oxigênio
FAL	Fosfatase Alcalina
5-FU	5-Fluoruoracila
GGT	Gama Glutamil Transpeptidase
HIF-1	Fator Induzido por Hipóxia
LDH	Lactato Desidrogenase
MHC	Complexo de Histocompatibilidade Principal
SOD	Superóxido Dismutase
TBARS	Substâncias Reativas ao Ácido Tiobarbitúrico
TCR	Receptor de Células T
T_{reg}	Linfócitos Reguladores ($CD4^+$ $CD45^+FOXP3^+$)

SUMÁRIO

INTRODUÇÃO

O Rio Grande do Sul é o estado brasileiro com a maior incidência de câncer cólon retal, apresentando uma incidência de 27,07 por 100.000 habitantes (BRASIL, 2010).

Para a escolha da melhor opção de tratamento, torna-se necessário determinar com precisão o estadiamento, com o objetivo de identificar não somente a extensão loco-regional da lesão primária, mas também a sua extensão à distância. Para tanto, o mesmo deve compreender todas as fases: pré, intra e também pós-operatória. Na identificação da extensão loco-regional da lesão primária, bem como a sua extensão à distância, é utilizado o estadiamento TNM. T refere-se à extensão do tumor, sendo T1 o menor grau, onde o tumor invade a submucosa e T4 o maior, quando o tumor invade diretamente outros órgãos ou estruturas; N refere-se ao comprometimento dos linfonodos pericólicos ou perirretais, sendo N0 a ausência de comprometimento dos linfonodos, N1 a presença de metástase em até 3 linfonodos e N2 presença de metástase em 4 ou mais linfonodos; M a presença de metástases à distância, sendo M0 ausência e M1 presença de metástases (GOSPODAROWICZ et al., 2004).

O câncer colón retal é potencialmente imunogênico e a resposta imune do paciente tem influência na sobrevida do mesmo. Vários mecanismos para a supressão imunológica têm sido descritos, afetando a imunidade inata e adaptativa, com a supressão ligada à pior evolução clínica (DIEDERICHSEN et al., 2003). A capacidade do sistema imune para controlar e moldar o câncer, conhecida como *cancer immunoediting*, é o resultado de três processos que ocorrem de forma independente ou em sequencia: eliminação (imunovigilância, no qual a imunidade funciona como supressor extrínsico do tumor, através da imunidade inata); equilíbrio (a expansão de células neoplásicas é mantida sob controle pela imunidade adaptativa); e escape (variantes de células tumorais com atenuada imunogenicidade ou com capacidade de atenuar as respostas imunes ocasionam um crescimento tumoral progressivo) (KOEBEL et al., 2007). As células T $CD8^+$ são fundamentais nesse processo, e sua quantificação nos tecidos tumorais apresenta valor prognóstico superior ao sistema de estadiamento TNM, tendo correlação com a sobrevida, independentemente da extensão da invasão tumoral, como demostrado esquematicamente na Figura 1 (GALON et al., 2007).

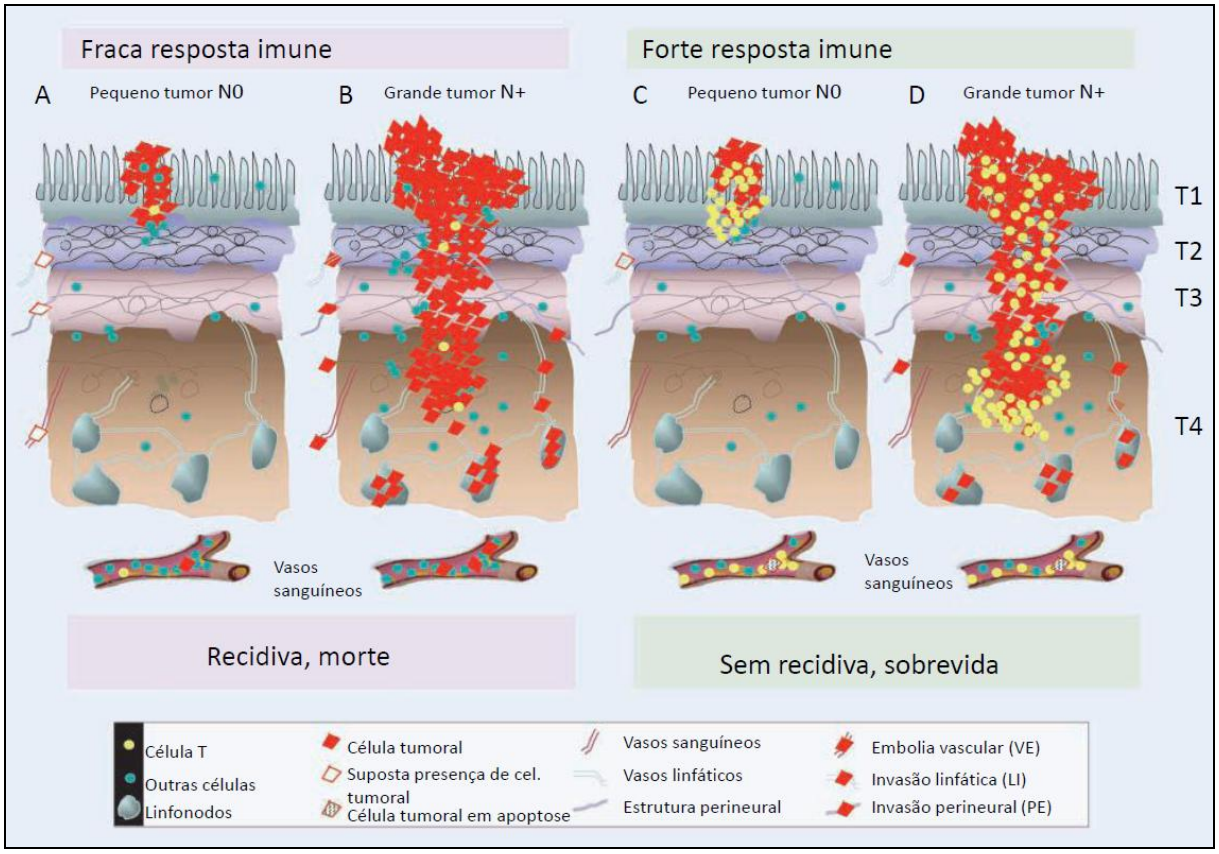

Figura 1 - Modelo de controle imune da disseminação do tumor cólon retal (adaptado de GALON et al., 2007)

Muitas células tumorais mostram um grande consumo de glicose, num fluxo rápido, com a maior parte do carbono derivado da glicose sendo secretado como lactato, e não como CO_2, apesar da disponibilidade de oxigênio. É o chamado Efeito Warburg. Há evidências que o Efeito Warburg é causado pela ativação do fator induzido por hipóxia (HIF-1), um fator de transcrição sensível às tensões de oxigênio, que regula centenas de genes envolvidos com muitos aspectos do metabolismo e da progressão dos tumores. A ativação do HIF-1 ocorre tanto sob condições de hipóxia, uma característica comum do microambiente do tumor, como também constitutivamente, pelo menos em alguns tipos de tumor. Essa ativação induz a glicólise aeróbica descrita por Warburg, através de coordenadas regulações enzimáticas: aumentando a expressão/atividade das enzimas glicolíticas e reduzindo o metabolismo oxidativo mitocondrial (STUBBS e GRIFFITHS, 2010; VANDER HEIDEN et al., 2009).

Essa adaptação metabólica exibida pelas células tumorais aumenta a produção de espécies reativas de oxigênio (EROs). EROs e o estresse oxidativo celular têm sido associados ao câncer a muito tempo. No entanto, a natureza dessa associação é complexa e às vezes pode parecer paradoxal. Sabe-se que: (1) estresse oxidativo e EROs podem causar câncer; (2) células transformadas parecem gerar mais EROs do que células normais; (3)

sistemas antioxidantes como tioredoxina e superóxido dismutase (SOD) encontram-se amplificados em células malignas; (4) a estimulação da progressão do ciclo celular, por fatores de crescimento ou por mutações que ativam a via de sinalização do receptor tirosina quinase, envolve um aumento de EROs; e (5) diversos agentes quimioterápicos podem ser seletivamente tóxicos para as células tumorais, porque eles aumentam o estresse oxidativo e levam estas já "estressadas" células para além do seu limite (SCHUMACKER, 2006).

As proteínas são as primeiras moléculas biológicas afetadas pelo estresse oxidativo nas células. Esse dano às proteínas pode ser mensurado pelo conteúdo de proteína carbonil e diversos estudos têm relatado níveis elevados em um número considerável de doenças, entre elas encontra-se o câncer **(**MALDONADO et al., 2005**;** MAYNARD et al., 2009**)**. Outra forma de caracterização do dano biológico causado por radicais livres é a peroxidação de lipídios, que pode ser avaliada pelo conteúdo de substâncias reativas ao ácido tiobarbitúrico (TBARS). Pacientes com câncer de colón retal possuem maiores níveis plasmáticos de TBARS que os indivíduos saudáveis (CHANDRAMATHI et al., 2009; SAYGILI et al., 2003).

De posse dos dados do estadiamento clínico dos pacientes com câncer cólon retal, o oncologista fará a escolha do esquema terapêutico adjuvante ou paliativo apropriado. Para os pacientes com comprometimento de linfonodos e/ou presença de metástases à distância é recomendado o esquema terapêutico denominado FOLFOX4, que consiste de 5-Fluoruoracila/ácido folínico + oxaliplatina durante 6 meses, com intervalo de 15 dias (ANDRÉ et al., 2004). O mecanismo de ação desses quimioterápicos resulta em danos ao DNA e o aumento dos radicais livres, que já estavam presentes nas células neoplásicas, em níveis superiores aos observados nos tecidos não transformados (LAURENT et al., 2005). O 5-Fluoruoracil (5-FU) inibe a divisão celular através do bloqueio da síntese do DNA (inibição da enzima timidalato sintetase) e pela formação de um RNA estruturalmente defeituoso (incorporação do 5-FU) (EMEA, 2009). Os efeitos da privação da síntese de DNA e RNA são mais marcados nas células de proliferação mais rápida e que metabolizam o 5-FU mais rapidamente. No entanto, à semelhança do que acontece com outros análogos dos nucleosídeos, o 5-FU é clastogênico nos linfócitos humanos (*in vitro)* (EMEA, 2009). Assim como outros derivados da platina, a oxaliplatina atua sobre o DNA, através da formação de ligações alquil que resultam no surgimento de pontes inter e intrafilamentos, inibindo sua síntese. A oxaliplatina induz a formação de ligações cruzadas entre duas guaninas adjacentes (GG) e entre adenina e guanina (AG) de fitas de DNA adjacentes, o que é comumente aceito como responsável pela sua citotoxicidade (ALMEIDA et al., 2006). A quantificação de danos

no DNA, induzidos por fatores relacionados a doenças e a identificação de substâncias tóxicas ou protetoras do DNA tornaram-se de vital importância. O Ensaio Cometa é muito utilizado para estimar os danos ao DNA, e foi descrito como sendo adequado para a detecção de ligações cruzadas induzidas por uma variedade de agentes químicos, dentre eles a oxaliplatina (ALMEIDA et al., 2006). Também permite de maneira simples e precisa a quantificação dos níveis de dano oxidativo ao DNA. Utilizando o Ensaio Cometa, foi demonstrado que as células do tecido neoplásico do cólon apresentam maior intensidade de dano oxidativo ao DNA do que as células oriundas do tecido normal (RIBEIRO et al., 2008)

A quimioterapia associa diferentes drogas buscando destruir as células tumorais, mas com efeitos indesejáveis por também atingir as células sadias. A quimioterapia induz leucopenia que pode ser recuperada mais rapidamente com Fatores Estimuladores de Colônia (CSFs), que são citocinas com a função de estimular a proliferação e a diferenciação dos precursores hematopoéticos (EMEA, 2009). A síntese dos CSFs, assim como seus receptores localizados na membrana das células pluripotentes, possui um rígido controle gênico. Além dos CSFs, as interleucinas (IL) são capazes de estimular a proliferação/maturação celular. Não há separação nítida entre as linhagens que respondem a ação dos CSFs e das IL. Ao contrário há sobreposição ou sinergismo de ação de vários fatores sobre a hematopoese, tanto sobre as células mais indiferenciadas, como as células precursoras medulares, como também sobre a diferenciação e a função das células mais maduras (ZAGO et al., 2004).

A *Uncaria tomentosa* (Willdenow ex Roemer and Shultes) DC., Rubiaceae, popularmente conhecida como unha de gato, é uma planta medicinal muito popular no Peru. Trata-se de uma trepadeira arbustiva que cresce apoiada em árvores, com folhas compostas, opostas e ovais. Seu nome foi inspirado na semelhança de seus espinhos com as unhas do gato. Nos países de língua inglesa é conhecida como Cat's claw. Mais de 50 constituintes químicos foram identificados, entre eles alcalóides oxindólicos e indólicos, polifenóis (flavonóides, proantocianidinas, taninos), glicosídeos triterpenos derivados do ácido quínico e quinóvico e saponinas (HEITZMAN et al., 2005). Entre estes, os alcalóides oxindólicos são reconhecidos como os marcadores fitoquímicos desta espécie por estarem associados com várias de suas ações farmacológicas (HEITZMAN et al., 2005; KEPLINGER et al., 1999; USP, 2009). A composição química pode variar de acordo com a região geográfica e época de coleta (HEITZMAN et al, 2005). Assim, algumas plantas apresentam conteúdos maiores de alcalóides oxindólicos tetracíclicos (AOT), outras alcalóides oxidólicos pentacíclicos (AOP). São AOP: Pteropodina (uncarina C), isopteropodina (uncarina E), especiofilina (uncarina D), uncarina F, mitrafilina, isomitrafilina. Os AOT encontrados na Uncaria tomentosa são:

rincofilina, isorincofilina, corinoxeina, isocorinoxeina. As estruturas dos AOP são apresentadas na figura 2.

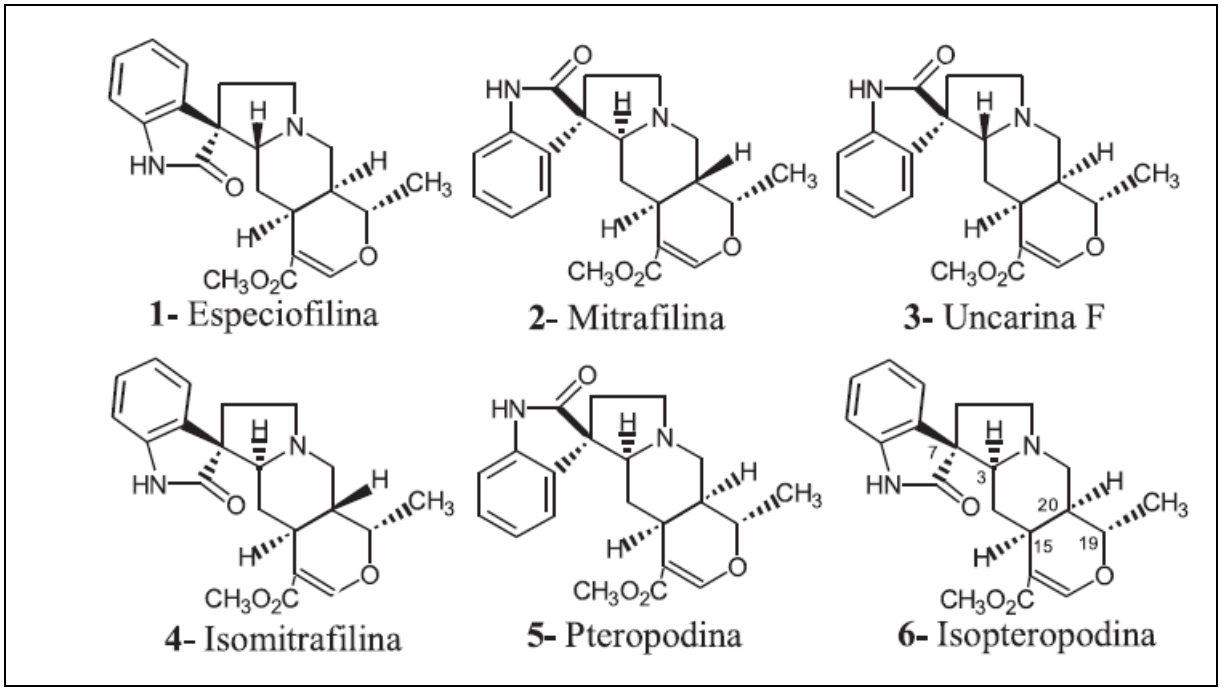

Figura 2 - Alcalóides pentacíclicos presentes no extrato de *Uncaria tomentosa* (adaptado de HEITZMAN et al., 2005)

Em relação à *Uncaria tomentosa*, diferentes extratos foram testados *in vitro* para determinar sua atividade antioxidante. Os extratos alcoólicos e aquosos de casca e raiz previnem a peroxidação lipídica e consequente dano à membrana e ao DNA (DESMARCHELIER et al., 1998; GONÇALVES et al., 2005). Em um estudo avaliando a atividade antioxidante global, o conteúdo de fenóis e a atividade da enzima antioxidante SOD, foi comprovada a alta capacidade antioxidante dos extratos de *Uncaria tomentosa* (PILARSKI et al., 2006). Em estudo com voluntários, houve diminuição no dano ao DNA e concomitante aumento no reparo do DNA no grupo que ingeriu o extrato aquoso de *Uncaria tomentosa* (C-MED 100®), quando comparado com grupo controle. Este ensaio clínico confirma resultados anteriores obtidos em modelo animal (ratos) com relação ao reparo de DNA mediado por C-MED 100® (SHENG et al., 2001).

Akeson et al. (2003) e Sheng et al. (2000a) em ensaio *in vivo* com ratas tratadas com extrato aquoso de *Uncaria tomentosa* encontraram um aumento dose-dependente no número de células no baço, mantendo as proporções normais de células T, B, *NK cells*, granulócitos e linfócitos de memória. Em estudo com voluntários, a utilização do extrato aquoso de *Uncaria*

tomentosa na dose de 5 mg/kg/dia por 6 semanas consecutivas elevou o número de leucócitos (SHENG et al., 2000a). Em estudo subseqüente, Sheng et al. (2000b) avaliaram o uso de extrato aquoso de *Uncaria tomentosa* (C-MED 100®) para tratar a leucopenia induzida por quimioterapia (doxorubicina) em modelo de ratos, usando Fator Estimulante de Colônia de Granulócitos (Neupogen®) como controle positivo. O tempo para a recuperação da leucopenia foi mais curto em ambos os grupos (C-MED 100® e Neupogen®) com relação ao grupo controle, sendo que C-MED100® recuperou todas as frações dos leucócitos de maneira proporcional, sugerindo um efeito mielo-estimulante direto. Esses dados foram confirmados em estudo realizado em 2005, por Eberlin e colaboradores, utilizando o modelo de ratos infectados com dose letal de *Listeria monocytogenes*. Estes autores analisaram a presença de fatores estimulantes de colônias (CSFs) no soro dos animais, avaliando sua capacidade de promover o crescimento e diferenciação de precursores hematopoéticos da medula óssea de animais normais. O pré-tratamento com 50 e 100 mg/Kg de extrato de *Uncaria tomentosa* potencializou a produção de CSFs, bem como produziu um aumento no número de CFU-GM medular quando comparado com o grupo controle. Também, foi observado aumento de IL1 e IL6 (AKESSON et al.,2003b). Pode-se concluir que os extratos de *Uncaria tomentosa* modulam a atividade imune e induzem aumento na reserva de precursores mielóides na medula óssea, em consequência da atividade biológica das citocinas liberadas (CSFs, IL1 e IL6).

Como o tratamento quimioterápico utilizando oxaliplatina e 5-FU é frequentemente restrito pelos efeitos tóxicos não seletivos sobre os tecidos normais, sendo a neutropenia a forma mais observada, torna-se necessário a busca de intervenções farmacológicas capazes de reduzir ou prevenir esses efeitos adversos. A *Uncaria tomentosa*, pelos seus efeitos mieloestimulantes, antioxidantes e de reparo ao DNA poderia ter um efeito positivo no tratamento do câncer cólon retal?

Neste contexto situam-se os objetivos deste estudo: (i) estudar alguns aspectos da fisiopatologia do câncer colón retal, envolvendo o sistema imune e o estresse oxidativo (ii) analisar o efeito da *Uncaria tomentosa* na recuperação da neutropenia induzida por quimioterapia, usando modelo animal e ensaio *in vitro,* (iii) e avaliar sua efetividade quando utilizada como adjuvante no tratamento do câncer colón retal, nos parâmetros hematológicos, imunológicos, estresse oxidativo e danos ao DNA.

ARTIGOS

Na sequência são apresentados os artigos que contém os resultados dos estudos realizados. São eles:

1. **ARTIGO 1- INFLUENCE OF PERIPHERAL BLOOD CD8$^+$ T CELLS ON CLINICAL OUTCOME OF CRC**, submetido ao **Scandinavian Journal of Immunology;**
2. **ARTIGO 2- CORRELATION BETWEEN TBARS LEVELS AND GLYCOLYTIC ENZYMES: THE IMPORTANCE TO THE INITIAL EVALUATION OF CLINICAL OUTCOME OF COLORECTAL CANCER PATIENTS**, **Biomedicine & Pharmacotherapy** v.65, p.95-400, 2011, doi:10.1016/j.biopha.2011.04.026;
3. **ARTIGO 3-** ***Uncaria tomentosa*** **STIMULATES THE PROLIFERATION OF MYELOID PROGENITOR CELLS**, **Journal of Ethnopharmacology** v.137, p. 856– 863, 2011, doi:10.1016/j.jep.2011.07.011;
4. ***ARTIGO 4- Uncaria tomentosa*** **FOR REDUCING SIDE EFFECTS CAUSED BY CHEMOTHERAPY IN CRC PATIENTS: CLINICAL TRIAL**. **Evidence-Based Complementary and Alternative Medicine,** v 2012, Article ID 892182, doi:10.1155/2012/892182.

ARTIGO 1- INFLUENCE OF PERIPHERAL BLOOD $CD8^+$ T CELLS ON CLINICAL OUTCOME OF CRC

Influence of peripheral blood CD8$^+$ T cells on clinical outcome of CRC

CD8 T cells and clinical outcome

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ABSTRACT

Tumor-infiltrating T cells have anti-tumor properties, in part because of the cytolytic activity of $CD8^+$ T cells. The objective of this study was to identify the relationship between $CD8^+$ T cell counts in peripheral blood with clinical outcome, taking into account CRC patient's TNM stage and gender. In addition, IL6serum levels were analyzed. Samples of blood and tumor tissue from 58 patients with colorectal cancer undergoing adjuvant/palliative chemotherapy were analyzed before and after six cycles of FOLFOX4. At the end of treatment, patients were grouped into two groups: poor outcome (PO) and good outcome (GO). Patients who had decreased $CD8^+$ T cell levels in peripheral blood, and therefore increased $CD4^+/CD8^+$ ratios, showed risk 2.19 for poor outcome. Women had fewer $CD8^+$ T cells than men in all TNM stages, and had risk 2.27 of poor outcome. The PO group showed increased IL6 levels after 6 cycles FOLFOX4. There was no correlation between $CD8^+$ T cell counts in peripheral blood and tumor tissue, but strong correlation with hemoglobin levels was observed. This may explain the relationships observed between women, anemia, low $CD8^+$ count and poor outcome.

Key words: T cells, survival, cancer, gender

INTRODUCTION

Colorectal cancer (CRC) is potentially immunogenic and the immune response of patients influences their survival [1]. Moreover, some anticancer drugs promote activation of the host immune system in addition to their direct cytotoxic effects on tumor cells, resulting in enhanced anti-tumor response [2]. Several mechanisms for immune suppression have been described, affecting the innate and adaptive immunity with suppression linked to poorer clinical outcome [3]. It has been shown that $CD8^+$ T cells and $CD4^+$ effector T cells (T_h) may have anti-tumor properties, whereas regulatory T cells ($CD4^+CD25^+$ T_{regs}) may be responsible for immunological hypo-responsiveness observed in cancer [4]. Consequently, the proportion of different T cell subtypes in lymphocytic infiltrate within tumors is reflected in clinical outcome. CRC patients with tumour infiltrating lymphocyte (TIL) presenting low $CD4^+/CD8^+$ T cell ratios have better clinical outcomes, with significantly higher survival at five years, regardless of stage of disease and age [3;5], because high density of effector memory T cells within the tumor are associated with decreased invasiveness, lower stage, and improved survival [6]. IL6 is also critical for T cell survival and differentiation. IL6 protein and mRNA are often upregulated in serum and tumor samples of humans and mice with breast, prostate, lung, liver, and colon cancer [7]. The proliferative and survival effects of IL6 are largely mediated by the oncogenic transcription factor STAT3, which has a profound impact on tumorigenesis [8].

However, there is little data available regarding the count of peripheral blood $CD8^+$ T cells and clinical outcomes of CRC cancer. The objective of this study was to identify the relationship between $CD4^+$ and $CD8^+$ T cell counts in peripheral blood with clinical outcome taking into account each CRC patient's TNM stage, gender. In addition, serum levels of IL6 were analyzed.

MATERIALS AND METHODS

Patients

The patients in this study were males and females with colorectal cancer who were undergoing adjuvant/palliative chemotherapy at the Oncology Service of the *Hospital Universitário de Santa Maria* of the *Universidade Federal de Santa Maria*, Brazil. Samples of blood and tumor tissue were collected from patients before the start of chemotherapy (n=58) and after six cycles of FOLFX4 (n=40). All patients had adenocarcinoma and had undergone surgery (colectomy) prior to the study. The tumor stage was determined by pathological examination of samples obtained at the time of resection and the presence of metastases by CT and X-rays, according to TNM-International Union Against Cancer [9].

At the end of treatment (12 cycles of FOLFOX4), patients were grouped according to their clinical response to treatment: poor outcome (PO) was designated as those patients whose computed tomography showed signs of metastasis (not present at diagnosis) without reductions or increases in the previous implants or CEA levels. Good outcome (GO) was defined as the inverse situation.

This project was approved by the Human Research Ethics Committee of the *Federal University of Santa Maria* (CAAE 0169.0.243.000-07), and all patients signed the Term of Free and Informed Consent.

Sample collection

Blood was collected in heparin and EDTA Vaccutainer® tubes before chemotherapy and after six FOLFOX4 cycles. The samples with EDTA were used for

hematologic parameters and immunophenotyping. The other samples were centrifuged (3000 rpm for 10 min), and the plasma was used to determine IL6 levels.

$CD3^+$, $CD4^+$, and $CD8^+$ Cells

Samples were collected in EDTA, and analyses were performed with a three-color fluorescence-activated cell sorter (FACScalibur, Becton Dickinson Biosciences, United States) and Multiset software (Becton Dickinson). FITC-conjugated anti-CD4, PE-conjugated anti-CD8 and PerCp-conjugated anti-CD3 were utilized. Immune subpopulations were measured as a percentage of the total number of $CD3^+$ cells.

Hemograms

Blood samples were analyzed in a Pentra apparatus (France). The values were confirmed by observation under optical microscopy of slides stained according to the May Grünwald-Giemsa method.

IL6

ELISA assays of IL6 were carried out according to a previously published method [10] at room temperature in 96-well microtiter plates (Nunc-Immuno Plate Maxi Sorp). Wells were coated with immunoaffinity-purified goat anti- IL6 antibodies (1 μg mL^{-1} in PBS, 100 μL) by incubation overnight at 4° C. The plates were washed three times with assay buffer (0.01 M Phosphate, 0.05 M NaCl, 0.1% Tween 20, pH 7.4). Samples or standards (100 μL) were added and incubated for 2 h. The plates were washed three times with 250 μL of buffer and incubated with biotinylated immunoaffinity-purified goat anti-IL6 antibodies (0.014 μg/ 100 μL/ well) for 1 h. The plates were washed three times with buffer and incubated with avidin-horseradish peroxidase (1/4000, 100 μL,

Sigma-Aldrich, St. Louis, MO, USA) for 15 min. The plates were washed three times with 250 µL buffer and incubated with o-phenylenediamine (OPD, 1 mM, Sigma-Aldrich, containing 0.4 µL of 30% v/v H_2O_2 mL^{-1}, 100 µL) for 15 min. The reaction was quenched with 150 µL 1 M sulfuric acid. Optical densities (O.D.) at 490 nm were determined using a microplate reader Thermo Scientific Multiskan FC (Vantaa, Finland).

Immunohistochemistry of infiltrating T cells

For histopathology and immunohistochemistry, paraffin-embedded sections were used. For immunohistochemistry, anti-CD8 primary antibodies were used (monoclonal mouse Anti-human, DAKO, clone C8/144.B). After deparaffinization, sections were pretreated by autoclaving for 5 min. The dextran-polymer method with MACH4 Universal HRP Polymer Kit (Biocare) was used as the secondary antibody. Each section had an area of approximately 1 cm^2. $CD8^+$ T cells were defined as $CD8^+$ T cells located within tumor tissue. No attempt was made to evaluate the various tumor compartments separately (eg, stroma, intraepithelial cells), and their distribution was quantified in ten independent microscopic fields (400x) for each patient sample to ensure representativeness and homogeneity. Areas near necrosis were excluded. Cell counting was performed using an ocular grid at 400X magnification. The counting was carried out by two independent observers, and the correlation coefficient between the mean of data obtained by the two observers was 78%. The results were expressed as the mean (± SD) number cells for one 400x microscopic field (0.0768 mm^2/field).

Statistics

Data were analyzed with EpiInfo program, version 3.5.1 from the CDC/USA. The data were evaluated by analysis of variance (ANOVA) and t-test, and are expressed as the mean ±SD. When the variances were not homogenous, ANOVA was not appropriate (Bartlett's P-value<0.05), and the Wilcoxon two-sample test was used to evaluate the data. P<0.05 was considered statistically significant. Pearson's correlation tests were performed in EXCEL 9.0 (Analyse-it v2.05).

The graphics of multivariate analysis were generated through principal component analysis (PCA) by the program Canoco 4.5. Due to the parameters evaluated, made up of different units, to compare them, was used a transformation of the data by ranking for each variable on a scale ranging from 1 to 5. The average value of the evaluated parameters corresponds to the scale of the number 2.5, with 1 being the lowest number corresponding to the evaluated parameter and 5 the highest.

RESULTS

Patient characteristics are shown in Table 1. All patients had undergone colectomy and were referred to the service for evaluation of adjuvant or palliative chemotherapy.

The absolute values of $CD8^+$ T cells ($CD3^+$, $CD8^+$, $CD4^-$) of peripheral blood showed significant differences between men and women, with average values of 415.2 cells/μL (limits of 96-817 cells/μL, SD=184.16) in women and 677.5 cells/μL (limits of 242-1533 cells/μL, SD=314.36) in men (P = 0.002). When stratifying patients according to TNM stage (International Union Against Cancer, UICC), there were significant differences in all stages; women showed lower values than men. The absolute values of $CD4^+$ T cells did not show a significant difference between stages of the disease (T, N,

and M) or between men and women. As shown in Table 2, the $CD4^+/CD8^+$ ratios were 1.69 (SD = 0.93) and 2.21 (SD = 0.83; P = 0.03) in men and women, respectively.

Of the 58 patients initially examined, it was possible to follow 44 patients who started adjuvant/palliative chemotherapy with FOLFOX4.

At end of the treatment, patients were grouped according to their clinical response to treatment: poor outcome (PO) or good outcome (GO). Seventy-six percent of patients with $CD4^+/CD8^+$ ratios > 2 were in the PO group, whereas 64% of patients with ratios <2 were in the GO group. The Risk Ratio (RR) was 2.12 (95% IC: 1.18 to 3.81, P=0.01) (Table 3).

According Diederichsen et al. (2003), patients with $CD4^+/CD8^+$ ratios > 2 have worse prognosis, significantly lower survival. The risk ratio (RR) analyze shows that the risk of having a $CD4^+/CD8^+$ ratio > 2 is 2.4 times more likely in women than in men (RR = 2.4375, 95% IC: 1.13 to 5.24, P = 0.01) (Table 4). When evaluating the likelihood of poor outcome in relation to gender, we found RR= 2.27 in women (95% IC: 1.03 to 4.96, P=0.01) (Table 5).

After six cycles of FOLFOX4, there were differences in $CD4^+$ T cell count between men in the PO and GO groups. There was no difference in T cell counts between groups after six cycles of FOLFOX4. The IL6 levels in the PO group were higher than those in the GO group before chemotherapy (14.48 versus 2.95 pg/mL, P<0.05) and after six cycles of FOLFOX4 (23.76 versus 4.01 pg/mL, P<0.0005). IL6 levels increased with disease progression in both PO groups (female and male), but there was no change in GO groups (Figure 1). There was no difference in IL6 levels between genders (10.35 pg/mL for females; 6.16 pg/mL for males) or TNM stages.

Immunohistochemical analysis of infiltrating T cells (Figure 2) revealed that the average number of $CD8^+$ T cells per field (x400) was 34.94 cells/field (± 16.7) in the

PO group and 48.96 cells/field (± 21.2) in the GO group. There was no difference between gender or TNM stage. Using the Pearson correlation, no correlation was found between $CD8^+$ T cell counts of tumor tissue and blood (R=-0.12).

Anemia, defined as hemoglobin ≤ 11 g/dL, was present in 42% of women and 12% of men. There is a positive correlation between $CD8^+$ T cells and anemia (R = 0.57, 95% IC: 0.33 to 0.75, P<0.0001) (Figure 3). When data were stratified according to clinical response, a higher correlation was found in the GO group (R=0.70) than in the PO group (R=0.47). In relation to $CD4^+$, this correlation is not present (R = 0.11, 95% IC: -0.2 to 0.40, P=0.471)(Figure 3).

The Hb levels varied significantly according to stage of disease in women but not in men. The average Hb level decreased as the extent of the tumor increased. Women in stages T1, T2, T3 and T4 showed Hb concentrations of 12.9 g/dL, 11.6 g/dL, 11.6 g/dL and 10.6 g/dL, respectively (P = 0.02). Similarly, women who presented metastasis at diagnosis had lower values of Hb compared to women without metastasis (10.8 g/dL versus 11.8 g/dL, P = 0.01). The graphics of multivariate analysis was made with the codes as follows: 1 = women included in the PO group, 2 = men included in the PO group; 3 = women included in the GO group, 4 = men included in the GO group. Multivariate analysis clearly shows the correlation between Hb levels and $CD8^+$ T cells, with high values related to men in both PO and GO groups (code 2 and 4). In the opposite case, the highest values of CD4/CD8 ratio related to women in the PO group (code 1) (Figure 3).

DISCUSSION

$CD8^+$ T cells, gender, cancer, and correlation with density in tumor tissue

The $CD4^+/CD8^+$ T cell ratio in peripheral blood is correlated with gender and age of healthy subjects due to variation in the absolute values of $CD4^+$ T cells; in contrast, the values of $CD8^+$ T cells are unchanged [11]. In our study, we found an inverse situation, where women had significantly lower values of $CD8^+$ T cells than men. Previous studies showed higher percentages of $FOXP3^+CD4^+$ T cells (T_{reg}) in healthy women compared to healthy men. Melanoma patients showed a 1.7-fold increase of T_{reg} cells in peripheral blood compared to healthy individuals, with women showing higher values [12]. Similar results were observed in a study with endometrial cancer patients, where circulating T_{reg} cells were more abundant in women with cancer compared to those without [13]. In addition, the proportion of T_{regs} in the peripheral blood of CRC patients is higher than that in normal controls [14]. However, there is little data about $CD8^+$ T cells in peripheral blood.

The elderly have lymphopenia and defective T cells. Aggarwal and Gupta [15] observed an increase in the expression of Fas and Fas ligand and decreased expression of Bcl-2 in both $CD4^+$ and $CD8^+$ T cells in elderly patients compared with young controls. These data suggest that increased apoptosis may be one of the mechanisms responsible for the deficiency of T cells associated with age. However, in our study, there is no significant difference in age between men and women. Thus, the reduction in the count of circulating $CD8^+$ T cells in women must be due to a factor other than age.

Salama et al. [16] showed that the density of $CD8^+$ $CD45RO^+$ cells in tumor tissue of CRC patients was lower than in normal tissue, and there was a correlation between the density of $CD8^+$ of the tumor tissue and normal tissue from the same patient. In this study, a correlation was not found between density of $CD8^+$ T cells in tumor tissue and circulating $CD8^+$ T cells count.

$CD4^+$, $CD8^+$ T cells and prognosis

The number of patients with $CD4^+/CD8^+$ ratio >2 was greater in the PO group (76.5% versus. 23.5% in GO group). However, when mean values were analyzed, there was no difference in $CD8^+$ T cell count according to clinical outcome. Perhaps because the SD were greater and/or because the immune response has a late effect in colorectal cancer, the prognostics of $CD8^+$ T cells in colon cancer may be more evident when the follow-up period is longer [17] than the time period used in our study. However, differences were observed with respect to $CD4^+$ T cells in peripheral blood of men, with higher values in the PO group.

A high density of $CD8^+$ T cells in normal and tumor tissue is associated with better survival, but T_{regs} cells have an ambiguous role. High counts of T_{regs} were associated with poor prognosis in normal mucosa but associated with improved survival in tumor tissue [16]. T_{regs} can suppress the proliferation of both CD4 and CD8 cells in co-culture experiments. T_{regs} suppress the activity of cytotoxic T cells through direct cell-to-cell contact or via the release of cytokines, especially transforming growth factor β (TGF β) [1;18]. Chen et al. [18] showed that $CD8^+$ cells failed to undergo normal functional maturation in the presence of T_{regs} because T_{regs} can effectively suppress the early tumor-specific immune response by $CD8^+$ cells by inhibiting their cytolytic activity in a TGF-β-dependent manner. In the present study, women had lower $CD8^+$ T cell counts than men and had a 2.27-fold higher risk for poor outcome. $CD8^+$ T cells are also essential in therapy response, as they are required to mediate chemotherapy, especially 5-fluoruoracil (5FU). 5FU exerts its activities through the elimination of Myeloid-derived suppressor cells (MDSC) and permits the restoration of T cell-dependent anti-tumor response. MDSCs have the ability to suppress T cell activation by inhibiting the antigen-specific reactivity of $CD8^+$ T cells through their capacities to

produce nitric oxide and radical oxygen species (ROS) [2]. In our study, the adjuvant/palliative CRC treatment with FOLFOX did not significantly alter the total count of $CD4^+$ or $CD8^+$ T cells.

IL6, T cells and prognosis

Tumor-infiltrating T lymphocytes strongly suppress tumor growth in the colon via inhibition of IL6 production through TGF-β production, thereby providing a novel mechanism for regulation of tumor cell growth by tumor-infiltrating lymphocytes [19]. IL6 levels were higher in the PO group before beginning chemotherapy. This difference increased during treatment in the group with disease progression (PO group), which could mean that T cells are not effectively inhibiting the production of IL6. This is consistent with data showing that IL6 enhances proliferation of tumor-initiating cells. The major pro-tumorigenic IL6 effector is the transcription factor STAT3, whose ablation in intestinal epithelial cells also results in decreased tumor multiplicity and growth [8].

Interestingly, it has been suggested that the sIL-6R controls the adherence of colon tumor cells to the vascular endothelium, thereby supporting the formation of metastases [19]. However, in the present study, there was no correlation between IL6 levels and levels of $CD4^+$ T cells or $CD8^+$ T cells in peripheral blood (data not shown).

Anemia and T cells

Cancer-related anemia is a cytokine-mediated disorder resulting from complex interactions between tumor cells and the immune system. Overexpression of certain inflammatory cytokines results in shortened survival of red blood cells, suppression of erythroid progenitor cells, impaired iron utilization, and inadequate erythropoietin

production. Numerous other factors may also contribute to the development of anemia in cancer patients [20].

Hb levels are linked with high cancer cell proliferation only in carcinomas overexpressing LDH5, which have poor vasculature. Hb levels and the resulting exogenous disposition of oxygen might play a major role in the activation of anaerobic pathways (energy acquisition by the transformation of pyruvate to lactate through LDH5 activity), resulting in more tumor aggressiveness [21]. In our study, there was no correlation between Hb and LDH, but there was a slight correlation between $CD8^+$ T cells and LDH in the PO group only (R=0.34, data not shown).

There was a positive correlation between $CD8^+$ T cell count in peripheral blood and Hb levels. One possible explanation for this observation may be related to hypoxia-adenosinergic inhibition of activity of $CD8^+$ T cells. Anemia can be associated with degree of oxygen supply, which results in local tissue hypoxia, and the stabilization of the HIF1α and hypoxia-driven accumulation of extracellular adenosine. The extracellular adenosine then signals via high affinity A2A adenosine receptors on the surface of $CD8^+$ and $CD4^+$ T cells. The hypoxia-adenosinergic inhibition, through A2A adenosine receptors on the surface of anti-tumor $CD8^+$ T cells, strongly inhibits T cell-mediated tumor rejection [22,23]. More studies are necessary to determine whether hypoxia-adenosinergic inhibition of activity of $CD8^+$ T cells could also interfere in proliferation of T cells. This could be the link among the four conditions observed in women: low Hb levels, low $CD8^+$ T cell counts, poor clinical outcome, and correlation between $CD8^+$ T cells and Hb levels.

CONCLUSION

The immunophenotyping of peripheral blood lymphocytes may help in understanding the differences in clinical outcome of patients with CRC. Women had a lower $CD8^+$ T cell count in peripheral blood than men and had a risk 2.27 times greater of a poor clinical outcome when treated with FOLFOX4. More studies are needed to determine with certainty the link among Hb levels, IL6, $CD8^+$ T cells, and clinical outcome observed in women.

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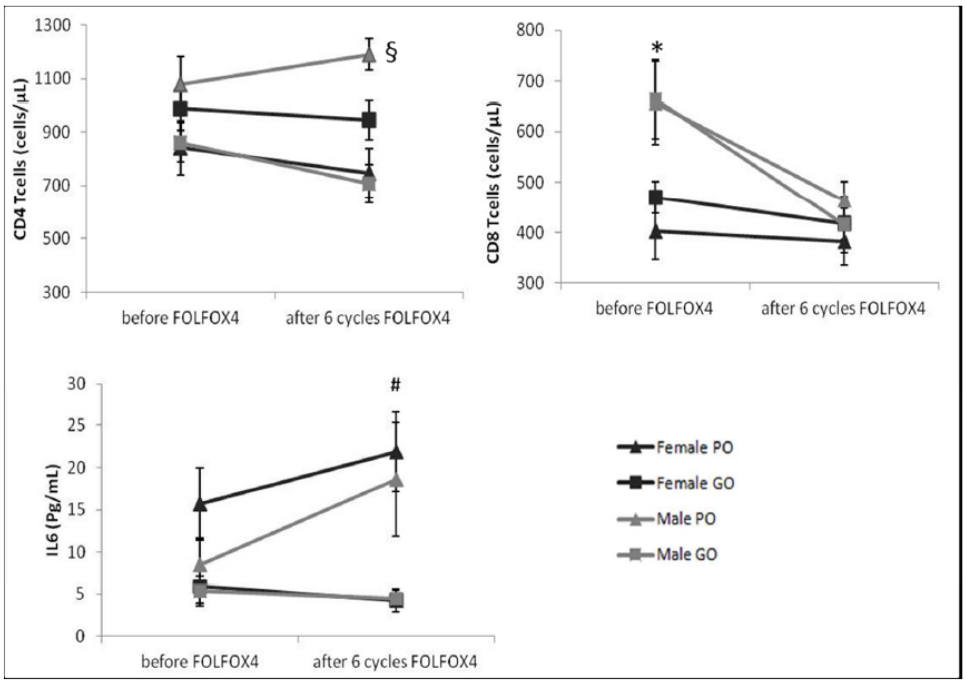
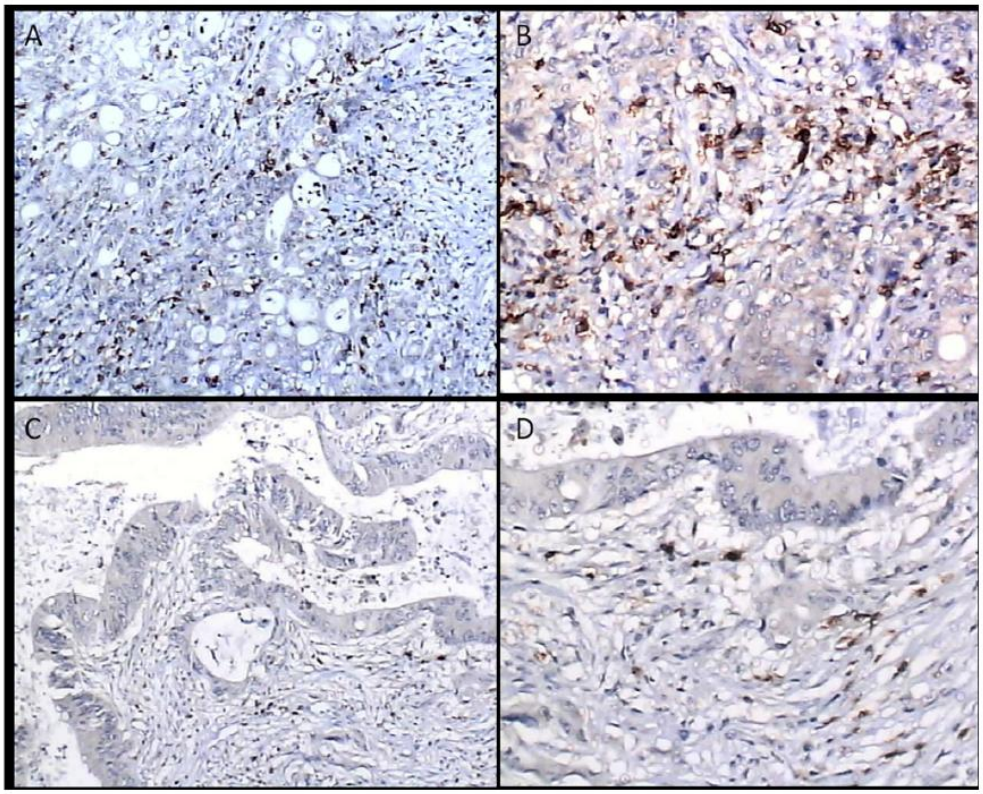
Figure 1



Immunophenotyping of peripheral blood lymphocytes of patients with CRC, grouped according to gender an clinical outcome, treated with FOLFOX 4.
Poor outcome group (PO) - was designated as those patients whose computed tomography showed signs o metastasis (not present at diagnosis) without reductions or increases in the previous implants or CEA levels Good outcome group (GO) was defined as the inverse situation. * Represents significant differences betwee genders; § represents differences as compared to before treatment; # represents differences between groups PO and GO (P<0.05). Data are expressed as the mean ±SE.

187x132mm (150 x 150 DPI)

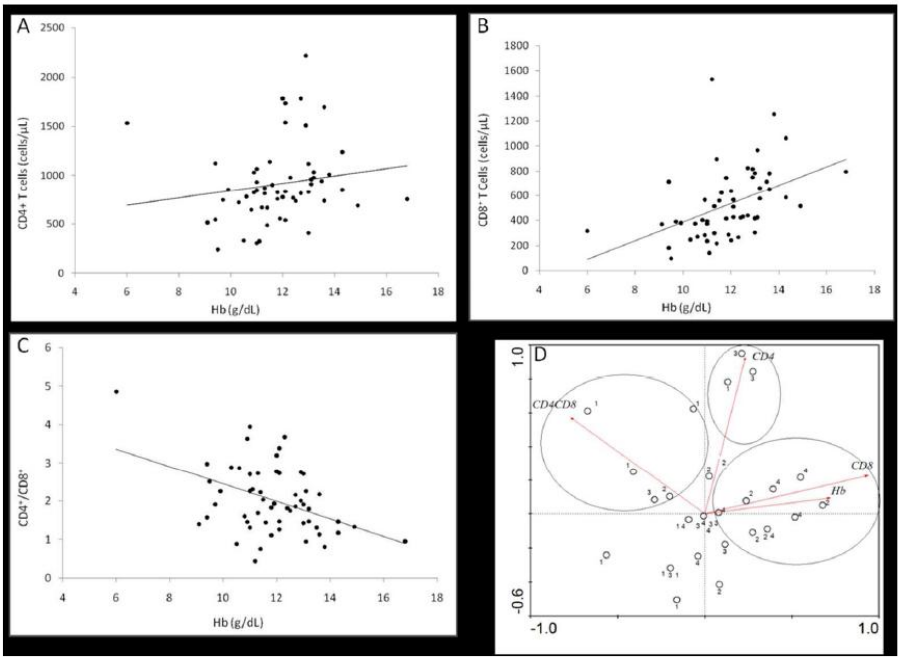
Figure 2



Density of the CD8+ T cells in colorectal tumor tissues
A and B, colorectal cancer sample with a high number of CD8+ T cells (brown staining) at 100 and 200 magnification respectively; C and D, colorectal cancer sample with low numbers of CD8+ T cells (brown staining) at 100 and 200 magnification respectively.

192x155mm (150 x 150 DPI)

Figure 3



Correlations between T cells in peripheral blood and hemoglobin levels of the CRC patients
A, correlation of the CD4+ T cells and hemoglobin levels; B correlation of the CD8+ T cells and hemoglobin levels; C, correlation of the CD4+ T/ CD8 + T cells ratio and hemoglobin levels; D multivariate analysis, where 1=women included in the PO group, 2= men included in the PO group; 3=women included in the GO group, 4= men included in the GO group

190x138mm (150 x 150 DPI)

Table 1- General characteristics of CRC patients.

		Female (n)	*Male (n*
Age	41-50 years old	6	5
	51-60 years old	10	6
	61-70 years old	10	8
	71-80 years old	7	6
T Stage	T1	1	0
	T2	1	1
	T3	21	20
	T4	10	4
N Stage	N0	1	5
	N1	16	8
	N2	13	12
M Stage	M0	17	20
	M1	14	4
Chronic diseases associated	Diabetes	3	4
	Hypertension	16	5
	Dyslipidemia	5	1
	Depression	3	3
	Other	1	7
n		33	25

Table 2 - Values of $CD4^+$ T cells, $CD8^+$ T cells and $CD4^+/CD8^+$ ratios of CRC patients, according to the TNM system.

		Female			*Male*	
TNM		T $CD4^+$ Cell/µL(±SD)	T $CD8^+$ Cell/µL(±SD)	CD4/CD8	T $CD4^+$ Cell/µL(±SD)	$TCD8^+$ Cell/µL(±SD)
T Stage	T3	1019.94 aA (511.66)	425.83 aB* (184.81)	2.48 aB (0.83)	1008.68 aA (339.26)	716.78 aA (335.79)
	T4	637.30 aA (232.26)	324.90 aB (150.50)	2.11 aA (0.64)	765.25 aA (177.44)	563.00 aA (162.12)
N Stage	N0				1271.25 a (1271.25)	746.50 a (109.77)
	N1	955.13aA (371.33)	459.66 aB (165.47)	2.22 aA (0.81)	976.25 aA (309.03)	817.50 aA (458.08)
	N2	934.61aA (546.89)	400.61 aB (189.16)	2.36 aB (0.85)	839.75 aA (162.84)	561.25 aA (197.69)
M Stage	M0	957.31aA (492.69)	445.43 aB (160.47)	2.20aB (0.77)	932.20aA (305.29)	694.65 aA (332.02)
	M1	805.60aA (459.27)	341.15 aB (191.38)	2.32 aA (0.90)	1256.00aA (393.15)	648.00 aA (243.10)

Different uppercase letter in the same row represents significant differences betw genders; different lowercase letters represent differences among stage (T, N, and the same gender. $P<0.05$

Table 3 - Prevalence of CRC patients with $CD4^+/CD8^+$ upper 2, in peripheral blood, according to clinical outcome.

		Clinical outcome		
		PO	GO	TOTAL
$CD4^+/CD8^+$	n	13	4	17
ratio>2	%	76,5	23,5	100,0
$CD4^+/CD8^+$	n	9	16	25
ratio<2	%	36,0	64,0	100,0
Total	n	22	20	42
	%	52,4	47,6	100,0

Table 4 - Prevalence of CRC patients with $CD4^+/CD8^+$ upper 2, in peripheral blood, according to gender.

		$CD4^+$ /$CD8^+$ Ratio		
		>2	<2	TOTAL
Female	n	18	14	32
	%	56,3	43,8	100,0
Male	n	6	20	26
	%	23,1	76,9	100,0
Total		24	34	58

Table 5 - Prevalence of women and men with CRC, according clinical outcome.

		clinical outcome		
		PO	GO	TOTAL
Female	n	18	9	27
	%	66,7	33,3	100,0
Male	n	5	12	17
	%	29,4	70,6	100,0
Total	n	23	21	44
	%	52,3	47,7	100,0

ARTIGO 2- CORRELATION BETWEEN TBARS LEVELS AND GLYCOLYTIC ENZYMES: THE IMPORTANCE TO THE INITIAL EVALUATION OF CLINICAL OUTCOME OF COLORECTAL CANCER PATIENTS

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Original article

Correlation between TBARS levels and glycolytic enzymes: The importance to the initial evaluation of clinical outcome of colorectal cancer patients

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ABSTRACT

Colorectal cancer (CRC) has been associated with high levels of lipid peroxidation, probably due to neoplasic tissue metabolism. Our objectives were to relate lipid peroxidation with the evolution of CRC and with various biomarkers (GGT, ALP, LDH, CEA) to assess its prognostic value. A longitudinal study was conducted with CRC patients ($n = 43$), using FOLFOX4. At the end of the treatment, patients were grouped into two groups: poor outcome (PO) for those patients whose computed tomography showed signs of metastasis, not reduced or increased in the previous implants, and not reduced or increased in CEA levels and good outcome (GO) for the opposite trends. PO patients had a significant increase in TBARS levels, being different from other group in cycles 4, 5, and 6 of chemotherapy. After cycle 6 of chemotherapy, GO patients had higher SOD (27%) and catalase (33%) activity. TBARS levels showed a positive correlation with biomarkers at the beginning of the treatment, which disappeared after six cycles of chemotherapy, when TBARS levels of the PO group started to increase; the other parameters increased at a later time. Because the serum TBARS levels in GO patients did not increase after the beginning of chemotherapy, it is expected that the increase is not a result of the effects of chemotherapy but of sickness evolution. It is possible that the systemic assessment of lipid peroxidation might become an additional marker because it occurs earlier than other biomarkers and could therefore be useful in the prognosis of CRC patients.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer in both men and women and the second most common cause of cancer death [1]. Proper staging is important for choosing the right treatment for a patient; the most clinically useful staging system is the tumor node metastasis (TNM) system maintained collaboratively by the American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC) [2]. Oxaliplatin is effective when combined with fluorouracil (FU) and leucovorin (LV) (FOLFOX) for treating metastatic colorectal cancer, and unequivocally improves disease-free survival (DFS), and is used in cycles of 15 days for a maximum of 12 cycles [3]. Biomarkers are used to support the diagnosis of cancer and are correlated with the prognosis. The most used biomarkers are carcinoembryonic antigen (CEA), lactate dehydrogenase (LDH), γ-glutamyltransferase (GGT), and alkaline phosphatase (ALP). It is possible that high serum CEA levels promote disease progression and metastasis rather than simply reflect a tumor burden increase [1]. LDH and GGT are related to the metabolic adaptation of cancer cells. Tumor cell proliferation requires rapid synthesis of macromolecules including lipids, proteins, and nucleotides. Warburg observed that proliferating tumor cells consume glucose at a high rate and release lactate instead of CO_2 [4]. Cancer cells undergo metabolic adaptation as a result of the expression of oncogenes such as Ras, Src or Bcl-Abl [6], which causes changes in genes that express particular enzymes (hexokinase2, TKTL1, PDK) [5], thus altering the mitochondrial activity [7,8]. This process is mainly initiated by the hypoxia inducible factor (HIF). A consequence of HIF activation is an increase in glucose uptake and phosphorylation due to elevated levels of both the glucose transporter Glut-1 and the glucose phosphorylating enzyme hexokinase [9]. Cells with a higher rate but lower yield of ATP may have a selective advantage when competing for shared energy resources [10]. The high glycolytic flux requires nicotinamide adenine (NAD^+), which may be efficiently generated from the conversion of pyruvate into

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lactate. This metabolic conversion makes glycolysis self-sufficient as long as high glucose uptake is possible [4]. Cellular ATP in tumors is mainly provided by oxidative metabolism; therefore, tumor cells do not rely on aerobic glycolysis for their energy needs [11]. The tricarboxylic acid (TCA) cycle is active and characterized by an efflux of substrates for use in biosynthetic pathways, particularly fatty acid synthesis. The success of this synthetic activity depends on the activation of pathways that generate reductive power (NADPH) and restore oxaloacetate for continued TCA cycle function (anaplerosis) [12]. Both these needs are met by the high rate of glutamine metabolism that transformed cells exhibit [5]. The end-product of glutaminolysis is, as for glycolysis, lactate, which is a product of pyruvate oxidation by LDH [4]. Also, glutamine is critical to maintaining the pool of glutathione, which is important for antioxidant defenses. The gamma glutamyl cycle, apart from explaining the synthesis and degradation of glutathione, may generate signals that promote active amino acid transport into cells [11]. Therefore, enzymes that catalyze reactions involved in glycolytic metabolism, glutaminolysis, and the gamma glutamyl cycle are important tools for monitoring and treating cancer.

Many types of cancer cells have increased levels of reactive oxygen species (ROS) [13]. A moderate increase in ROS can promote cell proliferation and differentiation whereas excessive amounts of ROS can cause oxidative damage to lipids, proteins, and DNA. The expression of genes associated with tumor transformation, such as Ras, Bcr-Abl and c-Myc, induce ROS production. In addition to oncogenic transformation, mitochondrial DNA (mtDNA) mutations have also been correlated to increased ROS levels in certain types of cancer cells, including those in solid tumors and leukemia. Several protein components of the electron transport chain are encoded by mtDNA. Thus, mutations of mtDNA are likely to cause impairments in electron transfer, leading to leakage of electrons and the generation of superoxide, which can subsequently be converted to other types of ROS [14]. Because an increase in ROS stress may induce oxidative damage of cellular components leading to cell death, cancer cells that are able to survive the intrinsic stress and develop a tumor must be equipped with sufficient adaptive mechanisms to tolerate the ROS stress. The adaptive processes involve activation of certain redox-sensitive transcription factors, which consequently lead to increased expression of the downstream genes encoding various ROS-scavenging enzymes and redox-sensitive survival machineries [15]. Thus, increased ROS stress in cancer cells is likely to increase expression of SOD and other antioxidant enzymes [16].

Lipid peroxidation occurs when ROS are generated close to or within membranes and attack the fatty acid side chains of membrane phospholipids. Similar to the increase of ROS, lipid peroxidation increases during the course of carcinogenesis in colorectal cancer [17].

In this paper, we analyzed the relationship between CEA, LDH, GGT, and ALP, which are commonly used as biomarkers in colorectal cancer, with oxidative stress and their roles as prognostic factors in colorectal cancer.

2. Patients and methods

2.1. Patients

The study was carried out with 43 patients (26 female, 17 male) that had undergone complete resection of histologically proven stage IIB, III and IV colorectal cancer and who were going to begin adjuvant/palliative chemotherapy with oxaliplatin + 5-fluorouracil/leucovorin (FOLFOX4) in the "Serviço de Hematologia e Oncologia of the Hospital Universitário de Santa Maria, Brazil". The control group consisted of 20 volunteers matched in age and gender to the patients.

At the end of the treatment, patients were grouped into two groups according to their clinical response to treatment: poor outcome (PO) for those patients whose computed tomography showed signs of metastasis (not present at diagnosis), no reduction or increase in the previous implants, and no reduction or increase in CEA levels; good outcome patients (GO) showed the opposite trends.

The Human Ethics Committee of the "Universidade Federal de Santa Maria" approved the study, and informed consent was obtained from all the participants (protocol number 0169.0.243.000-07).

2.2. Sample collection

Blood was collected in citrated, EDTA and without anticoagulant Vaccutainer tubes before chemotherapy and after each of the six cycles. CAT and SOD activities were determined on a sample of whole blood that had been diluted 20 fold in saline solution.

2.3. Biomarkers

A COBAS INTEGRA system was used to quantify the catalytic activity of LDH, GGT, and ALP; data were acquired using a COBAS INTEGRA 400 plus apparatus (USA).

LDH was measured with a UV assay; GGT activity was measured with an enzymatic colorimetric assay; ALP activity was measured using a colorimetric assay. CEA levels were measured by the IMMULITE 2000 Kit, a solid-phase, two-site sequential chemiluminescent immunometric assay, using an IMMULITE 2000 Analyzer (USA).

2.4. Carbonylation of serum protein

The carbonylation of serum proteins was determined by a modified Levine's method [18]. The absorbance of the supernatant at 370 nm was measured using a spectrophotometer. Carbonyl content was calculated using $22 \times 10^3 mM^{-1}\,cm^{-1}$ as the molar extinction coefficient, and the results were expressed as nanomoles of carbonyl groups per milligram protein.

2.5. Determination of lipid peroxidation

Lipid peroxidation was estimated by measuring TBARS levels in plasma samples according to a modified method of Jentzsch et al. (1996) [19]. The concentration of malondialdehyde (MDA) was determined by measuring the absorbance at 532 nm using a spectrophotometer. The results were expressed as nanomoles of MDA per milliliter of plasma.

2.6. Catalase (CAT) and superoxide dismutase (SOD) activities

CAT activity was determined in accordance with a modified method of Nelson and Kiesow (1972) [20]. The change in absorbance at 240 nm was measured for 2 min. CAT activity was calculated using the molar extinction coefficient ($0.046\,mM^{-1}\,cm^{-1}$), and the results were expressed as picomoles of CAT per milligram of protein.

SOD activity was determined based on the inhibition of the radical superoxide reaction with adrenaline as described by McCord and Fridovich (1969) [21]. SOD activity is determined by measuring the rate of adrenochrome formation, observed at 480 nm, in a medium containing glycine-NaOH (50 mM, pH 10) and adrenaline (1 mM).

2.7. Statistics

Data were analyzed with EpiInfo program, version 3.5.1 from the CDC/USA [22]. The data were evaluated by analysis of variance (ANOVA) and *t*-test, and are expressed as the mean ± SEM. When the variances were not homogenous, ANOVA was not appropriate (Bartlett's P value < 0.05), and the Wilcoxon two-sample test was used to evaluate the data. $P < 0.05$ was considered statistically significant. Pearson's correlation tests were performed in EXCEL 9.0 (Analyse-it v2.05).

3. Results

Most patients were aged between 50 and 65 years ($n = 22$); 15 patients had upper age, (65–80 years), and only six were younger than 50 years. The characteristics of CRC patients included in this study, according TNM system (T-size and extent of the primary tumor; N- involvement of regional lymph node; M- presence or absence of distant metastases), were as follows: T1 = 1; T2 = 2; T3 = 28; T4 = 12; N0 = 3; N1 = 16; N2 = 22; M0 = 30; M1 = 13 subjects.

At the end of the treatment, patients were divided into two groups: those who had a good treatment outcome (GO group) and those who had adverse outcomes (PO group). It was possible to assess 39 patients; one patient died due to unrelated illness; two patients transferred to other services, and one patient withdrew from the treatment. Twenty patients were grouped in the GO group and 19 patients in the PO group. Nine out of these 19 patients passed away while other showed disease progression. Disease progression was due to the emergence of metastases during treatment in six of the patients.

Serum levels of ALP, GGT, and CEA decreased at the beginning of chemotherapy. LDH was the only parameter that increased in the GO group. After cycle 12, all biomarkers were significantly different between the PO and GO groups (Fig. 1).

All patients had high levels of lipid peroxidation, measured as MDA levels, which represent TBARS levels, at all analyzed stages (before the start of chemotherapy and after every six cycles). These values were four times higher than those in the volunteer control

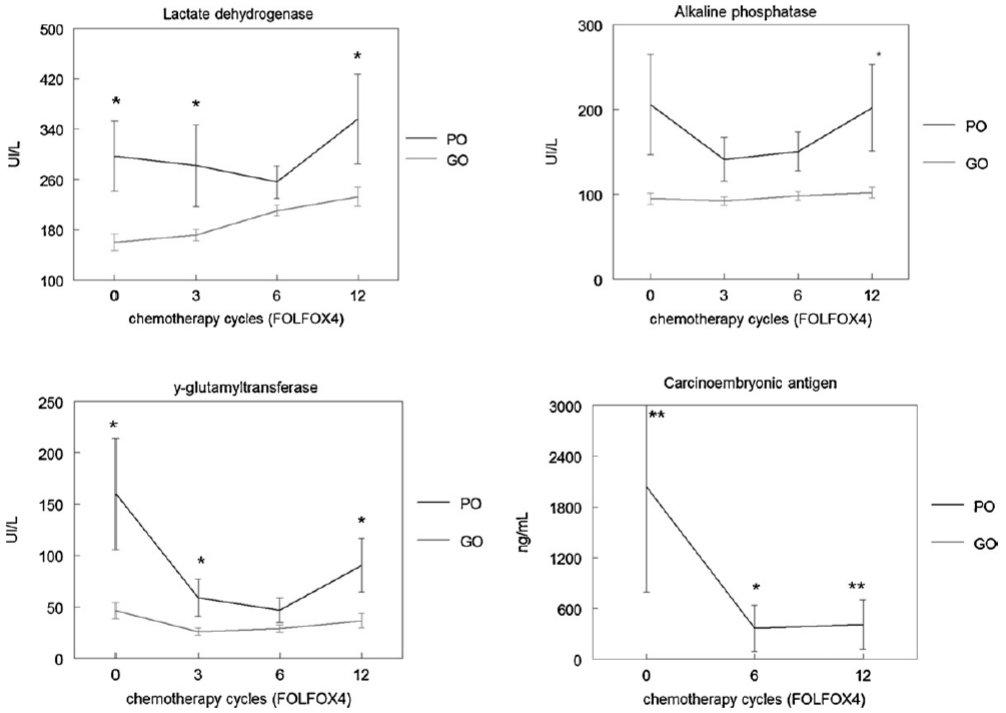

Fig. 1. Variations of biomarkers of the colorectal cancer (CRC) patients according clinical outcome. Serum levels of lactate dehydrogenase (LDH), alkaline phosphatase (ALP), y-glutamyltransferase (GGT), and carcinoembryonic antigen (CEA) were analyzed during 12 cycles of adjuvant/palliative chemotherapy (FOLFOX4) in patients with CRC. Patients were grouped into two groups: poor outcome (PO) for those patients whose computed tomography showed signs of metastasis (not present at diagnosis), not reduced or increased in the previous implants, and not reduced or increased in CEA levels; good outcome (GO) the opposite situation. The values represent the mean ± SEM. An asterisk (*) denotes $P \leq 0.05$; (**) denotes $P \leq 0.001$ statistical differences between groups using analysis of variance followed by *t*-test.

Table 1
Stress oxidative and antioxidant defense of colorectal cancer (CRC) patients in adjuvant/palliative chemotherapy (FOLFOX4).

	Control	Before chemotherapy	After cycle 3 FOLFOX	After cycle 6 FOLFOX
TBARS nmol MDA/mL	4.95 ± 1.66	20.01 ± 10.98***	18.78 ± 9.04***	22.34 ± 10.97***
Carbonyl protein nmol/mg protein	0.44 ± 0.17	0.70 ± 0.31**	0.67 ± 0.29**	0.73 ± 0.32**
Catalase pmol/mg protein	6.97 ± 2.02	8.19 ± 4.13	8.19 ± 3.98	9.38 ± 3.98*
SOD U/mg protein	1.39 ± 0.39	1.87 ± 0.68*	2.17 ± 0.68**	2.25 ± 0.66***,a

TBARS: thiobarbituric acid-reactive substances; SOD: superoxide dismutase.
*$P < 0.05$; **$P \leq 0.001$; ***$P \leq 0.0001$, as compared to the control group.
[a] As compared to before chemotherapy: $P < 0.05$.

Table 2
Stress oxidative and antioxidant defense of colorectal cancer (CRC) patients in adjuvant/palliative chemotherapy (FOLFOX4) according TM stage.

	T			M	
	T2	T3	T4	M0	M1
Before chemotherapy (FOLFOX4)					
TBARS nmol MDA/mL	13.75 ± 1.06	20.99 ± 11.6	19.63 ± 11.4	18.15 ± 9.58	24.03 ± 13.5
Catalase pmol/mg protein	6.31 ± 0.96	8.53 ± 4.82	7.87 ± 3.24	8.06 ± 4.75	8.13 ± 3
SOD U/mg protein	1.68 ± 1.4	1.83 ± 0.66	1.92 ± 0.56	1.78 ± 0.65	1.96 ± 0.68
After cycle 6 FOLFOX4					
TBARS nmol MDA/mL	12.84 ± 9.7	21.44 ± 9.72	26.67 ± 12.9	20.69 ± 10.5	25.48 ± 11.6
Catalase pmol/mg protein	8.63 ± 5.34	9.73 ± 4.4	8.72 ± 3.27	9.2 ± 4.13	9.79 ± 3.76
SOD U/mg protein	2.83 ± 0.46	2.05 ± 0.63	2.4 ± 0.58	2.27 ± 0.62	2.18 ± 0.78

TBARS: thiobarbituric acid-reactive substances; SOD: superoxide dismutase.

group. Patients with colorectal cancer also had higher values of protein carbonylation and activity of the antioxidant enzyme SOD compared to control subjects. Throughout chemotherapy treatment, there was a significant increase in SOD activity ($P = 0.016$) (Table 1).

No difference in TBARS, SOD, or catalase was observed in relation to tumor staging (T-tumor spread; N-involvement of regional lymph node, and M- presence/absence of metastases at diagnosis). Stage T1 was not included in the statistical analysis because it had only one patient (Table 2).

Even though there was no difference related to oxidative stress based on tumor size and presence of metastases, an important difference was observed in the prognosis of these patients. For example, TBARS levels increased early in the PO group. These differences become significant after the 4th cycle of chemotherapy (Fig. 2). There was no difference in TBARS levels during the treatment in the GO group, demonstrating that lipid peroxidation was not due to the toxicity of chemotherapy but to the progress of the disease.

Two patients showed higher TBARS levels without any increase in other biomarkers, true for hyperglycemia (grade 4 and 3, according to CTCAE 4.03). These patients were part of the GO group, indicating the need to care for this interfering factor.

After six cycles of chemotherapy, patients in the GO group showed an increase in the activity of SOD (27%) and catalase (33%)

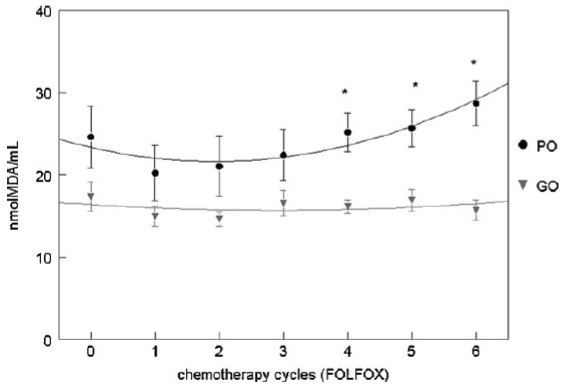

Fig. 2. TBARS levels of the colorectal cancer (CRC) patients in adjuvant/palliative chemotherapy with FOLFOX4, according clinical response to the treatment. Lipid peroxidation was evaluated by TBARS concentration, and expressed as nmol MDA/mL (mean ± SEM). Data were collected before each cycle of chemotherapy of the two groups patients: poor outcome group (PO) for those patients whose computed tomography showed signs of metastasis (not present at diagnosis), not reduced or increased in the previous implants, and not reduced or increased in carcinoembryonic antigen (CEA) levels; good outcome group (GO) the opposite situation. An asterisk (*) denotes $P \leq 0.001$ statistical differences between groups using analysis of variance followed by *t*-test.

Table 3
Pearson's correlation between biological markers colorectal cancer (CRC) and stress oxidative at day zero (before chemotherapy).

	Carbonyl	SOD	CAT	ALP	GGT	LDH	CEA
TBARS	0.51**	−0.03	0.16	0.58***	0.59***	0.67***	0.46*
Carbonyl		0.29	0.18	0.39*	0.44*	0.4*	0.37*
SOD			0.15	−0.1	−0.07	−0.07	−0.12
CAT				−0.08	−0.05	−0.11	−0.15
ALP					0.96***	0.78***	0.90***
GGT						0.86***	0.85***
LDH							0.64***

TBARS: thiobarbituric acid-reactive substances; CAT: catalase; SOD: superoxide dismutase.
*$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$.

Table 4
Pearson's correlation between oxidative stress and biomarkers colorectal cancer (CRC) after Cycle 6 FOLFOX.

	Carbonyl	SOD	CAT	ALP	GGT	LDH	CEA
TBARS	−0.3	−0.29	−0.03	0.04	−0.15	0.16	0.1
Carbonyl		0.21	0.004	0.12	0.15	−0.22	0.04
ALP					0.8***	0.56**	0.75***
GGT						0.52**	0.69***
LDH							0.68***

TBARS: thiobarbituric acid-reactive substances.
**$P < 0.001$, ***$P < 0.0001$.

($P < 0.05$) while no significant changes were observed in the other group.

TBARS levels provided a correlation between parameters of oxidative stress and other biomarkers of colorectal cancer at diagnosis (Table 3).

However, this correlation is not observed after six cycles of chemotherapy; rather, the only correlations observed are between the biomarkers (Table 4). Analyzing CEA, ALP, GGT, and LDH during treatment demonstrates that after six FOLFOX cycles, these biomarkers did not increase in patients in the PO group, which only occurred in subsequent cycles (Fig. 1). These differences in systemic response explain the lack of correlation between TBARS levels with other biomarkers in the sixth cycle of treatment.

4. Discussion

4.1. Biomarker of colorectal cancer

LDH, GGT, ALP, and CEA are commonly used in clinical medicine as biomarkers of colorectal cancers. In the 1950s, studies had already shown an increase in glycolytic enzymes (LDH and glucose phosphate isomerase) in patients with colorectal cancer [23]. A significant increase in GGT and ALP has initially been related to

liver and bone metastases, respectively. Subsequently, high levels of GGT and ALP in patients were correlated with a worse prognosis even though detectable metastases were not present in these organs [24]. ALP as well as CEA, albumin, and clinical stage had significant prognostic value for crude survival in patients with CRC liver metastases [25]. LDH is possibly the most studied of these enzymes for the use as a cancer biomarker. Several studies have linked the expression of LDH with advanced stages of cancer and poor overall survival [26]. LDH-5 is directly regulated by HIFs, which as noted above, are activated by ROS. LDH activity has an important impact on mitochondria bioenergetics and interferes with the proliferation of tumor cells. LDH and mitochondria activity are mutually regulated at the level of metabolites. They depend on the availability of pyruvate and the NADH/NAD+ ratio. The fast kinetics of the LDH-catalyzed conversion of pyruvate into lactate ensure a rapid and constant supply of ATP but rapidly consumes NADH. LDH regenerates NAD+ in the cytoplasm and competes with NADH transport into the mitochondria and subsequently with its oxidation [27]. Thus, upregulation of LDH provides a highly efficient anaerobic/glycolytic metabolism for tumor cells [26]. Koukourakis et al. (2006) [26] have observed that there was a relationship between LDH-5 expression in tumor specimens and LDH serum levels. Therefore, increased levels of serum LDH in the present study are due to LDH-5. Our data are consistent with those from previous studies; in the PO group there was a significant increase in LDH levels compared to the GO group before the beginning of chemotherapy, with an increasing difference from cycle 6 of FOLFOX.

There are no studies that show the exact relationship between GGT and the metabolic adaptation of tumor cells, although the importance of the glutamine and glutathione cycles is clear, in which GGT is involved. Tumor hypoxia stimulates the expression of γ-glutamylcysteine and glutathione transferase, resulting in high intracellular glutathione levels, which correlate with high proliferation and resistance to anti-cancer treatments [5]. GGT is essential for the transport of glutamine, which is required for the growth of many types of cells. Therefore, supplementation with glutamine might stimulate neoplastic proliferation, promoting a more anaplastic, less differentiated phenotype and interfering with integrin-mediated adhesion [28]. Previous studies have shown the relationship between glutathione S-transferase (GSTP1) and HCT116 human colon cancer cells. GSTP1 protects cells from oxidative stress by avoiding apoptosis and promoting cell survival and proliferation [29]. Regarding ALP, although several studies have shown that cancer patients have high levels of the enzyme, which has a predictive value for recurrence being a very sensitive indicator of the progression of cancer with liver metastasis [30], there are no studies demonstrating its relationship with tumor cell metabolism and oxidative stress.

4.2. Lipid peroxidation

Levels of malondialdehyde (MDA), a sensitive marker of radical-mediated lipid peroxidation, were higher in the serum of all colorectal carcinoma patients than in control samples. Hendrickse et al. (1994) [31] and Özdemirler et al. (1998) [32] have observed higher levels of MDA in patients with colorectal cancer, which has also been observed in cancerous tissue samples of the cancer. Lauschke et al. (2002) [33] have found differences in TBARS levels according to the T-stage and observed higher serum lipid peroxides with increased tumor stage. A relationship was found due to the sampling in which, unlike a previous study, all patients included in the study had clinical stage III and IV tumors, with only three patients in stage T1 and T2 according to the TNM classification. The two patients in stage T2 had the lowest TBARS levels; if there had been a larger number of patients in the early stages of the disease, the differences may have been significant. On the other hand, the data indicate the relationship between an increase in TBARS levels and disease progression. The aforementioned authors concluded that tumors are the cause of increased systemic lipid peroxides because lipid peroxide levels diminished when the malignant tissue was successfully resected. We may assume that the opposite is true, where levels increase with disease progression.

High levels of TBARS are consistent with previous observations that have detected high levels of free radicals, both O_2 and H_2O_2, in *in vitro* assays with CT26 (mouse colon carcinoma cells) [34].

Various results have been found when the mitochondria are isolated from tumor tissue to determine the content of TBARS. When the lipid peroxide levels were available in mitochondria obtained from tumors, these values were the same compared to adjacent normal tissues of subjects with colorectal cancer [35]. It is important to consider the metabolic adaptation of the tumor cell, in which a more oxidizing environment, as a result of greater cell metabolic activity [36], is partially compensated by the induction of antioxidants [37]. Transformed cells use ROS signals to drive proliferation and other events required for tumor progression [38]. Laurent et al. (2005) [34] assessed the origin of ROS, *in vitro*, using tumor cells and fibroblasts. In nontransformed cells, ROS were low; ROS originating from NADPH oxidase and H_2O_2 were controlled by the glutathione system. In tumor cells, there were high levels of ROS, close to the threshold of cytotoxicity, which were produced by the mitochondria, and H_2O_2 was controlled by catalase. Mitochondrial MnSOD, but not CuZnSOD [16,35] or GSH-Px [35], activity increases in malignant tissues; therefore, mitochondria may be protected from the oxidative stress produced by excess O_2 and H_2O_2. Superoxide is efficiently decomposed by MnSOD to H_2O_2, which is freely diffusible. In the cell cytosol, H_2O_2, likely in combination with other free radical species, can create a more oxidizing redox state, which activates HIF [37]. Higher MnSOD levels in colorectal carcinomas contribute to tumor cell resistance and therapy insensitivity, resulting in a poor clinical outcome [16]. It is possible that the more oxidizing redox state of the cytosol explains the high TBARS levels in PO patients (metastases). In our study, we observed a slight increase in SOD activity in colorectal cancer patients compared to the control group. The levels of antioxidants and SOD do not necessarily reflect the redox state of a cell. High cellular antioxidant levels commonly occur in cells undergoing oxidative stress, with the more oxidizing cell redox state acting as a stimulus for antioxidant induction. Apparently, in a contradictory way, the activities of both SOD and CAT slightly increased throughout the treatment with oxaliplatin, being more intense in patients with a better clinical response, which may have contributed to minimizing lipid peroxidation in this group.

4.3. Correlation between lipid peroxidation and cancer biomarkers

Because LDH, GGT, and ALP levels are proportional to the amount of tumor tissue [24], it is possible that the initial decrease in the levels of LDH, ALP, GGT and CEA are due to removal of the tumor (colectomy) and that increases from cycle 6 are due to the proliferation of metastatic cancer cells, explained by the metabolic adaptation of tumor cells. Pearson's correlations between TBARS, LDH and GGT showed a significant relationship between oxidative stress and these glycolytic enzymes, which are used as biomarkers of cancer. The same positive relationship was observed for ALP, but more research is necessary to identify their interaction in aerobic/glycolytic metabolism. Our data confirm the results by Munjal et al. (1976) [24] who observed correlations between the enzymes (LDH and GGT) and CEA similar to the values we found ($r = 0.68$ and 0.58). Our research is the first to show a significant correlation between TBARS and various biomarkers at the beginning of chemotherapy. After six cycles of FOLFOX4, this positive correla-

tion no longer existed. Analyzing the graphs with biomarkers serum levels, there was an increase in biomarkers in the PO group, in which LDH and GGT only begin to increase after cycle six, and ALP increases slightly earlier (Fig. 1). TBARS levels increase after the third cycle, earlier than the biomarkers. Lauschke et al. (2002) [33] elucidated another important aspect in TBARS research: the sensitivity of the technique to identify cancer in early stages. Specific tumor markers only significantly increase in late stages, not in early stages (T1 or T2), while systemic lipid peroxidation was significantly higher at any stage of the tumor.

5. Conclusion

We conclude that TBARS levels could be used as an early marker of colorectal cancer progression at a stage in which other markers have not yet shown significant changes. Also, TBARS levels can be used in conjunction with established tumor markers to help in the early diagnosis of colorectal cancer. The strong correlation of TBARS levels with the overexpression of enzymes involved in the glycolytic metabolic adaptation of tumor cells (GGT, LDH) indicates that the observed increase in TBARS levels in the group with disease progression is due to the increase in ROS generated by this high metabolic flux. It is noteworthy that small metastases that still have not been detected by imaging (CT) result in a measurable change in lipid peroxidation. Other enzymes or products of glycolytic metabolism may also be useful for the early detection of colorectal cancer progression, thus helping to ensure that the most appropriate chemotherapy treatment is used.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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ARTIGO 3- *Uncaria Tomentosa* STIMULATES THE PROLIFERATION OF MYELOID PROGENITOR CELLS

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Uncaria tomentosa stimulates the proliferation of myeloid progenitor cells

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ABSTRACT

Ethnopharmacological relevance: The Asháninkas, indigenous people of Peru, use cat's claw (*Uncaria tomentosa*) to restore health. *Uncaria tomentosa* has antioxidant activity and works as an agent to repair DNA damage. It causes different effects on cell proliferation depending on the cell type involved; specifically, it can stimulate the proliferation of myeloid progenitors and cause apoptosis of neoplastic cells. Neutropenia is the most common collateral effect of chemotherapy. For patients undergoing cancer treatment, the administration of a drug that stimulates the proliferation of healthy hematopoietic tissue cells is very desirable.

It is important to assess the acute effects of *Uncaria tomentosa* on granulocyte-macrophage colony-forming cells (CFU-GM) and in the recovery of neutrophils after chemotherapy-induced neutropenia, by establishing the correlation with filgrastim (rhG-CSF) treatment to evaluate its possible use in clinical oncology.

Materials and methods: The *in vivo* assay was performed in ifosfamide-treated mice receiving oral doses of 5 and 15 mg of *Uncaria tomentosa* and intraperitoneal doses of 3 and 9 μg of filgrastim, respectively, for four days. Colony-forming cell (CFC) assays were performed with human hematopoietic stem/precursor cells (hHSPCs) obtained from umbilical cord blood (UCB).

Results: Bioassays showed that treatment with *Uncaria tomentosa* significantly increased the neutrophil count, and a potency of 85.2% was calculated in relation to filgrastim at the corresponding doses tested. An *in vitro* CFC assay showed an increase in CFU-GM size and mixed colonies (CFU-GEMM) size at the final concentrations of 100 and 200 μg extract/mL.

Conclusions: At the tested doses, *Uncaria tomentosa* had a positive effect on myeloid progenitor number and is promising for use with chemotherapy to minimize the adverse effects of this treatment. These results support the belief of the Asháninkas, who have classified *Uncaria tomentosa* as a 'powerful plant'.

1. Introduction

Uncaria tomentosa belongs to the Rubiaceae family and is commonly known as cat's claw. It is a woody vine native to the Amazon rainforest and other tropical areas of South and Central America. This plant has been used in traditional and cultural practices in South America for centuries, especially in Peru. References exist regarding indigenous groups such as the Asháninkas who use preparations of 'powerful plants' to restore health, and *Uncaria tomentosa* is one of these plants (Keplinger et al., 1999). Today, this plant is widely used in Peruvian traditional medicine as an anti-inflammatory, contraceptive, and cytostatic remedy (Aquino et al., 1991). It has been used in patients with rheumatic diseases

Abbreviations: BFU-E, burst forming units-erythroid; CFC assay, colony-forming cell assays; CFU-E, colony-forming units-erythroid; CFU-GEMM, colony-forming unit-granulocyte/erythrocyte/macrophage/megakaryocyte; CFU-GM, colony-forming unit-granulocyte/macrophage; CSFs, colony stimulating growth factors; GSH, glutathione; GM-CSF, granulocyte/macrophage colony-stimulating factor; hHSPCs, human hematopoietic stem/precursor cells; IL, interleukin; NPSH, non-protein thiols; NF-kappa B, nuclear factor kappa B; POAs, pentacyclic oxindole alkaloids; ROS, reactive oxygen species; SOD, superoxide dismutase; TOAs, tetracyclic oxindole alkaloids; TNF-α, tumor necrosis factor α; UCB, umbilical cord blood; WBC, white blood cell.

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(Mur et al., 2002) or cancer (Gonzales and Valerio, Jr., 2006) due its antioxidant (Pilarski et al., 2006) and anti-inflammatory properties (Allen-Hall et al., 2007). *In vitro* and *in vivo* studies using animal models have also demonstrated that it has DNA repair activity (Sheng et al., 2000a). Furthermore, micromolar concentrations of its purified pentacyclic oxindole alkaloids (POAs) inhibit the growth of neoplastic cells. For example, mitraphylline can induce apoptosis of human Ewing's sarcoma (MHH-ES-1) cells and breast cancer cells (MT-3 cells) (García Gimenez et al., 2010), pteropodine and uncarine F induce apoptosis of human lymphoblastic leukemia T cells (CCRF-CEM-C7H2) (Bacher et al., 2005), and mitraphylline induces apoptosis of human glioma (Gamgee cell) and neuroblastoma cells (SKN-BE) (Garcia Prado et al., 2007). Finally, these compounds stimulate the proliferation of myeloid precursors (Eberlin et al., 2005).

Neutropenia is the most common collateral effect of chemotherapy and may be a limiting factor in determining the frequency and dose of treatment. Patients with neutropenia are predisposed to infection due to an absence of granulocytes; the disruption of the integumentary, mucosal, and mucociliary barriers; and the inherent microbial flora shifts following severe illness and antimicrobial usage (Ozer et al., 2000). Colony stimulating growth factors (CSFs), especially for the myeloid lineage (G-CSF), are occasionally used to minimize the effects of neutropenia. They are part of the family of cytokines that regulate the proliferation, differentiation, and functional activation of myeloid hematopoietic cells. Filgrastim (recombinant human granulocyte colony-stimulating factor-rh-G-CSF) is a glycoprotein produced by recombinant DNA technology in *Escherichia coli*. Secondary administration of CSFs may allow chemotherapy dose maintenance, which is important because it improves overall survival, disease-free survival, quality of life, non-toxicity, and cost-effectiveness (Ozer et al., 2000).

For patients undergoing cancer treatment, the administration of a drug that stimulates the proliferation of healthy hematopoietic tissue cells is desirable because it could minimize the primary adverse effects of chemotherapy. *Uncaria tomentosa* has these characteristics, which make it a promising auxiliary to conventional treatments for cancer. Some studies have demonstrated the positive effect of *Uncaria tomentosa* on leukocyte counts over a period of eight weeks in healthy animals (Sheng et al., 2000a) and after 10 days of mild doxorubicin-induced neutropenia (Sheng et al., 2000b). It is important to observe its acute effect on myeloid tissue using a model of severe neutropenia and to assess whether the results obtained with the animal model can be extrapolated to humans (Gertsch, 2009). These observations are necessary to determine possible therapeutic and toxic doses (Keplinger et al., 1999). This study was conducted to investigate the effect of *Uncaria tomentosa* on myeloid progenitors. The objectives of this study were as follows: (1) to establish the dose-dependent relationship between *Uncaria tomentosa* and a reference drug (Filgrastim) on myeloid cell proliferation after ifosfamide-induced neutropenia; (2) to evaluate the interference of *Uncaria tomentosa* with reactive oxygen species (ROS) production using animal models; and (3) to assess the relationship of *Uncaria tomentosa* with cell proliferation. The result was confirmed using colony-forming cell (CFC) assays with human hematopoietic stem/precursor cells (hHSPCs).

2. Materials and methods

2.1. Drugs

Filgrastim (Leucin®, Bergamo, São Paulo, Brazil), ifosfamide (Glenmark, São Paulo, Brazil), and ascorbic acid (Redoxon®, Bayer, Brazil) were obtained. The *Uncaria tomentosa* extract was prepared by ultra-turrax extraction (Biotron-Kinematica AG) of ground bark (Centroflora) with 70% ethanol (Dipalcool). The fluid was centrifuged (Centrifuge Suzuki), concentrated in a heating tank (MCA-ALW) to remove the alcohol, and spray-dried (Kohls) using silicon dioxide (Evonik) and microcrystalline cellulose 102 (Blanver) as excipients.

2.2. Analysis of dry Uncaria tomentosa extract

The following reagents were used: acetonitrile (JTBaker), triethylamine (Fluka), acetic acid (JTBaker), polyamide (Fluka), ethanol (Vetec), and ultrapure water. Sample extraction was performed using a Unique® ultrasound, model USC 5000A, 40 kHz. Chromatographic analyses were performed on the Agilent 1100 HPLC system and a Zorbax® XDB C-18 column (150 mm × 4.6 mm, 3.5 μm Agilent) at 15 °C. Samples (80 mg) were diluted in 60% ethanol (10 mL) and subjected to sonication (20 min at 30 °C). Next, 2 mL of sample were passed through a column containing 200 mg of polyamide, and the eluate was injected into an HPLC system. Separation was achieved using a gradient elution of water (0.2% acetic acid) adjusted to pH 6.9 with triethylamine (A) and acetonitrile (B) at a flow rate of 0.8 mL/min. The composition of the mobile phase was 65% of A and 35% of B in the 0–18 min interval; a linear gradient approaching 50% of A and 50% of B in 18–29 min; 50% of A and 50% of B in 29–31 min; a linear gradient approaching 65% of A and 35% of B in 31–32 min; and 65% of A and 35% of B in 32–38 min. Each run was followed by an equilibration period of 6 min. Detection was performed at 245 nm, and the concentration of pentacyclic oxindole alkaloids (POA) were calculated with reference to external calibration curves of mitraphylline (Bertol, 2010).

2.3. Laboratory animals

Male 7- to 8-week-old BALB/c mice from the Central Animal House of the Federal University of Santa Maria (UFSM) were housed in air-conditioned controlled conditions (room temperature 22 ± 2 °C and relative humidity of 65%; artificial illumination, 12 h per day) and were used when they weighed between 19 and 24 g. They were given food and water *ad libitum*. All animal procedures were approved by the Animal Ethics Committee of the *Universidade Federal de Santa Maria*.

2.4. Biological assay

The bioassay was performed as previously described (Dalmora et al., 2006). The animals were allocated to *Uncaria tomentosa*, filgrastim reference standard, and control groups in a fully randomized method and were identified by color code for the assay. Each group consisted of 6 mice per treatment group. Standard and test samples were diluted to the concentrations of 6 and 18 μg/mL and 10 and 30 mg/mL, respectively, with phosphate buffered saline containing 0.1% bovine serum albumin. A single dose of 200 mg of ifosfamide/0.5 mL per mouse was injected intraperitoneally into each animal on day 0. Multiple intraperitoneal injections of 0.5 mL of filgrastim and oral doses of *Uncaria tomentosa* were given to the ifosfamide-treated mice from day 1 to day 4. Six hours after the last injection/administration, peripheral blood was collected from the orbital venous sinus. Blood films were prepared on glass slides and stained using the May–Grünwald–Giemsa method, in which the neutrophils were counted and expressed as a percentage of the total number of white cells.

Another bioassay was performed as previous described, with animals allocated into two groups: ascorbic acid and control. Ascorbic acid was diluted to the concentrations of 11 mg/mL according Jagetia et al. (2003). Multiple oral doses of 0.5 mL of ascorbic acid were given to the ifosfamide-treated mice from day 1 to day 4.

2.5. Hematological analysis

The white blood cell (WBC) count was performed in a Neubauer chamber (optical, 100×) in duplicate, and the blood films were stained with May–Grünwald–Giemsa for light microscopy observation. The hemoglobin concentration was determined using Drabkin's solution at a spectrophotometric absorbance of 540 nm. The samples were analyzed by two different individuals.

2.6. Antioxidants

Non-protein thiols in the plasma were assayed and evaluated with the Ellman's method (1959). Values are expressed in mmol/mL of protein. The determination of catalase activity in blood was carried out in accordance with the modified method of Nelson and Kiesow (1972). This assay involves the changes at an absorbance of 240 nm for 2 min due to the catalase-dependent decomposition of hydrogen peroxide (H_2O_2). The enzyme activity was calculated using the molar extinction coefficient (0.0432 cm^{-1} μmol^{-1}). The results were expressed in picomoles/mg protein. Superoxide dismutase (SOD) activity measurements were based on the inhibition of the superoxide radical reaction with adrenaline as described by Mc Cord and Fridovich (1969). In this method, SOD present in the sample competes with the detection system for superoxide radicals. The units of SOD are defined by the amount of enzyme that inhibits 50% of adrenaline oxidation. The oxidation of adrenaline leads to a colored product, adrenochrome, and is then detected by spectrophotometry. SOD activity is determined by measuring the speed of adrenochrome formation, observed at 480 nm, in a reaction medium containing glycine-NaOH (50 mM, pH 10) and adrenaline (1 mM).

2.7. Allometry calculation

To determine the mammalian metabolic rate, we used the following formula: $(MR) = aM_b{}^b$, with $a = 3.98$, $b = 0.686$, and M_b = body mass in grams (White and Seymour, 2005).

2.8. Colony-forming cell (CFC) assays

CFC assays were performed with hHSPCs obtained from umbilical cord blood (UCB). UCB was collected with full term deliveries ($n = 2$), and written informed consent was given by all mothers. All procedures were performed sequentially, immediately after collection and in aseptic conditions. The sample was diluted 1:3 in PBS, and the mononuclear cells were separated with density gradient solution polysucrose and diatrizoate sodium (Histopaque-1077, Sigma–Aldrich) following the manufacturer's instructions. Absolute and differential counting was performed (equipment Sysmex Xs1000i) in the cells obtained at the interface of mononuclear cells, and their viability was assessed by the trypan blue assay (Sigma–Aldrich). A positive selection was performed later using immunomagnetic separation for $CD34^+$ progenitors on the Dynal® CD34 Progenitor Cell Selection system (Invitrogen™ Dynal®) according to the manufacturer's instructions. $CD34^+$ cells were obtained from hemocytometer counting and assessed for viability using blue trypan before the CFC assays were carried out.

CFC assays were performed using semi-solid Complete MethoCult® H4434 (methylcellulose, HSCF, HGM-CSFhIL-3, hEPO StemCells Technologies) according to the manufacturer's instructions for human hematopoietic CFC assays (human colony-forming cell assays using MethoCult®, Technical Manual, version 3.0, October 2004). The *Uncaria tomentosa* extract was added to the culture medium at a final concentration of 100 and 200 μg/mL before the addition of $CD34^+$ cells. The medium was vortexed, and 7.5×10^2 $CD34^+$ cells were added per 1.1 mL of medium. Then, 1.1 mL of medium (7.5×10^2 cells) was transferred to each 35-mm plate. Cultures were incubated at 37 °C under 5% CO_2 in air and humidity higher than 95% for 15 days. Subsequently, they were visualized under a microscope with phase contrast. The tests were performed in two independent experiments and in duplicate for controls and tests.

The trial was approved by the Human Research Ethics Committee of the Universidade *Federal de Santa Catarina*, Protocol number 311/2008, in November 2008.

2.9. Statistical analysis

Statistical analyses of the bioassay data were carried out according to Finney by parallel line methods (2×2) using a PLA 2.0 Program (Stegmann Systemberatung, Rodgau, Germany) to calculate the potency and confidence intervals ($P = 0.05$). Analysis of variance was performed for each assay, and the assumption of regression and parallelism of the log dose–log response lines were tested ($P = 0.05$). The data for hematological analysis were analyzed with the EpiInfo program, version 3.5.1, from the CDC/USA. The data were evaluated by analysis of variance and *t*-test and are expressed as the mean ± SD.

3. Results

3.1. Pentacyclic oxindole alkaloids from Uncaria tomentosa extract

The HPLC analysis of dry *Uncaria tomentosa* extract has a content of 2.57% POA. The concentrations of each POA were as follows: speciophylline – 0.26%; uncarine F – 0.07%; mitraphylline – 0.80%; isomitraphylline – 0.40%; uncarine C – 0.46%; and uncarine E – 0.58%. The chromatographic profile in Fig. 1 does not include the tetracyclic oxindole alkaloids (TOA), rhynchophylline and isorhynchophylline.

3.2. Hematological evaluations

The experimental mice receiving filgrastim and *Uncaria tomentosa* showed significant recovery of their neutrophil counts compared to the control group after ifosfamide-induced neutropenia. Additionally, the recovery (increase) of neutrophils was four and thirteen times higher for the two doses of *Uncaria tomentosa* tested, respectively. The minimum neutrophil count for the dose of 15 mg per mouse was 593.7 cells/μL, whereas the control group showed 0.0 cells/μL, with the highest value of 211.5 cells/μL. Thus, the dose of 15 mg of *Uncaria tomentosa* showed positive qualitative effects near the normal range, while the leukocytes of the control group were almost entirely lymphocytes (91.7%), as shown in Table 1 and Fig. 2. Additionally, the values obtained for the control group showed the utility of the experimental model of ifosfamide-induced leukopenia.It is essential to establish dose dependency with a well-established drug in clinical use in studies with herbal remedies because they are extracts instead of purified, active compounds. Therefore, a bioassay was performed to compare a biopharmaceutical sample of 3 and 9 μg doses of filgrastim and 5 and 15 mg of *Uncaria tomentosa*. The validity of the assay was shown by the analysis of variance, and the results were statistically calculated, giving a potency of 85.20%.

Ascorbic acid was used as a positive standard, in order to evaluate antioxidant activity. The use of ascorbic acid at dose of 5.5 mg per mouse (250 mg/kg) resulted in leukocytes count increase (4493.7/μL, SD 1545.9, $P = 0.02$) when related to control (2255.0/μL, SD 724.6). But, different from the *Uncaria tomentosa* or filgrastim treatment, the major increase occurred in lymphocyte

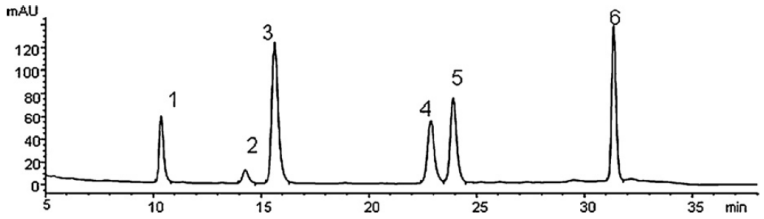

Fig. 1. HPLC-fingerprint analysis of dry extract from *Uncaria tomentosa*. Speciophylline (1), uncarine F (2), mitraphylline (3), isomitraphylline (4), uncarine C (5) and uncarine E (6).

Table 1
Recovery of leukocytes and hemoglobin values with *Uncaria tomentosa* (VO) or filgrastim (IP) four days after induction of leukopenia with ifosfamide (IP) in mice.

Group	Control	*Uncaria tomentosa*		Filgrastim	
Dose	X $n=6$	5 mg VO $n=6$	15 mg VO $n=6$	3 μg IP $n=6$	9 μg IP $n=5$
Leucocytes (cell/μL)	2025.6^{A} (1267.6)	2230.0^{A} (1114.5)	2690.1^{A} (1261.1)	3395.1^{A} (2933.7)	4125^{A} (1933.5)
Neutrophils (cell/μL)	98.3^{C} (85.4)	464.4^{B} (255.1)	1366.5^{AB} (605.7)	1571.9^{A} (1408.9)	1979.5^{A} (1672.2)
Lymphocytes (cell/μL)	1919.5^{A} (1180.0)	1608.7^{A} (840.4)	1164.9^{B} (544.8)	1637.7^{A} (1440.4)	1849.8^{A} (358.1)
Monocytes (cell/μL)	27.2^{C} (20.4)	166.8^{AB} (180.2)	158.6^{B} (129.8)	185.4^{A} (136.0)	289.6^{A} (190.3)
Hemoglobin (g/dL)	12.7^{A} (1.9)	14.38^{A}a (1.8)	15.55^{A} (1.5)	13.87^{A} (1.6)	14.95^{A} (2.3)

Values expressed as means (SD). Values in the same row that do not share the same upper case superscript letters are significantly different among the treatments.

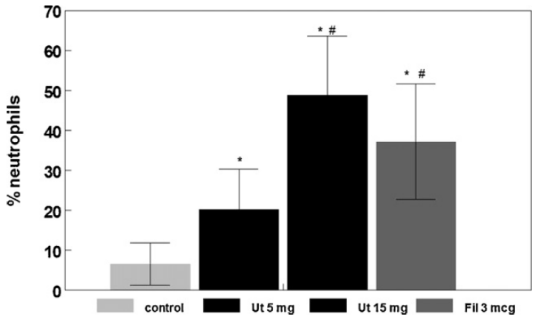

Fig. 2. Recovery of neutrophils (% values) with *Uncaria tomentosa* (VO) or filgrastim (IP) four days after induction of leukopenia with ifosfamide (IP) in mice. $^{*}P<0.005$ in relation to the control group; $^{\#}P<0.05$ between treatments (Ut 15 mg × Ut 5 mg; Fil 3 μg × Ut 5 mg); data were represented as means ± SD. Ut 5 mg = *Uncaria tomentosa* dry extract 10 mg/mL; dose 0.5 mL/day per animal; Ut 15 mg = *Uncaria tomentosa* dry extract 30 mg/mL; dose 0.5 mL/day per animal; Fil 3 μg = 6 μg/mL, dose 0.5 mL/day per animal.

count (3257.9/μL, SD 88.4 vs 1873.0/μL, SD 543.6 in the control group, $P=0.01$).

3.3. Oxidative stress

There were no differences in levels of non-protein thiols or in the activities of antioxidant enzyme catalases or superoxide dismutase (SOD) among *Uncaria tomentosa*, filgrastin, and control groups (Table 2).

Table 2
Values of antioxidants in animals treated with *Uncaria tomentosa* or filgrastim, after ifosfamide-induced neutropenia.

	Control	*Uncaria tomentosa* 15 mg	Filgrastim 3 μg
NPSH [a] (μmol SH/mL)	0.73 (0.08)	0.75 (0.04)	0.73 (0.05)
SOD[b] (UI/mg of protein)	9.09 (0.11)	7.11 (1.92)	8.41 (1.66)
Catalase (nmol/mg of protein)	9.00 (1.64)	8.81 (0,90)	7.71 (1.64)

Values expressed as mean ± SD.
[a] Non-protein thiols.
[b] Superoxide dismutase.

The experimental mice receiving ascorbic acid showed mild reduction (33%) on non-protein thiols levels in relation to the control mice, four days after induction of leukopenia with ifosfamide. There were no differences in antioxidant enzymes (data not shown).

3.4. Allometry scale

To evaluate whether the dose tested in the animals is suitable to be used in humans, the allometric scale was used (White and Seymour, 2005). Allometry is the study of how a dependent variable (metabolic rate) varies in relation to an independent variable (body mass). The allometric comparison allows one to calculate dosages and frequencies of drug administration to individuals based on individual calorie needs. The *Mammalian basal metabolic rate* (MBR) for this animal study (mice) was 35.2 mL O_2 h^{-1}; for humans, it was 7545.6 mL O_2 h^{-1}. Thus, considering that the average weight of animals was 24 g, mice treated with 0.5 mL of solution containing 10 mg of dry *Uncaria tomentosa* extract received 208 mg/kg of *Uncaria tomentosa* that corresponded to a dose of 17.8 mg/kg or 1 g/day for a 60-kg human.

3.5. In vitro assay

CFC assays showed possible increases in cell proliferation after *Uncaria tomentosa* extract administration *in vitro*, evidenced by the overplating effect observed in these cultures. This effect did not occur in the control trials conducted simultaneously with the same cell samples. The overplating effect made it impossible to accurately count the number of colonies, making it undesirable for data collection; therefore, we opted for a semi-quantitative and qualitative assessment. Semi-quantitatively, the observed overplating effect was caused by an increase in the size of granulocyte-macrophage colony-forming cells (CFU-GM) and mixed colonies (CFU-GEMM), presenting considerable confluence/overlap (Figs. 3 and 4). Qualitatively, a reduction in the intensity of the reddish color given by the hemoglobin to the B-FUE colonies was observed. This reduction was more evident at the concentration of 200 μg extract/mL, which suggests lower hemoglobinization of these colonies in cultures with *Uncaria tomentosa*, as seen in Fig. 5. Images are representative of a semi-quantitative and general

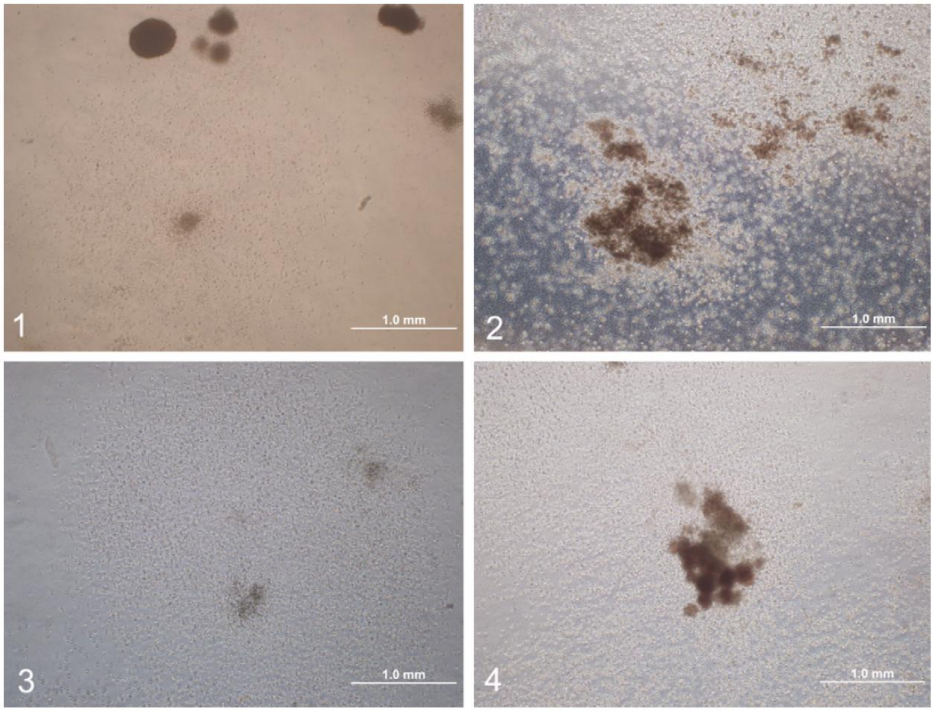

Fig. 3. CFC assays treated with extract of *Uncaria tomentosa*, final concentration of 100 μg of extract/mL medium (40×). Photo 1: CFC assay controls showing the absence of overplating (40×). Photo 2: demonstration of the overplating effect with the addition of *Uncaria tomentosa* extract. Photos 3 and 4: CFU-GM and CFU-GEMM, respectively, with high granulocytic proliferation, occupying nearly the entire microscopic field in extract-treated cell.

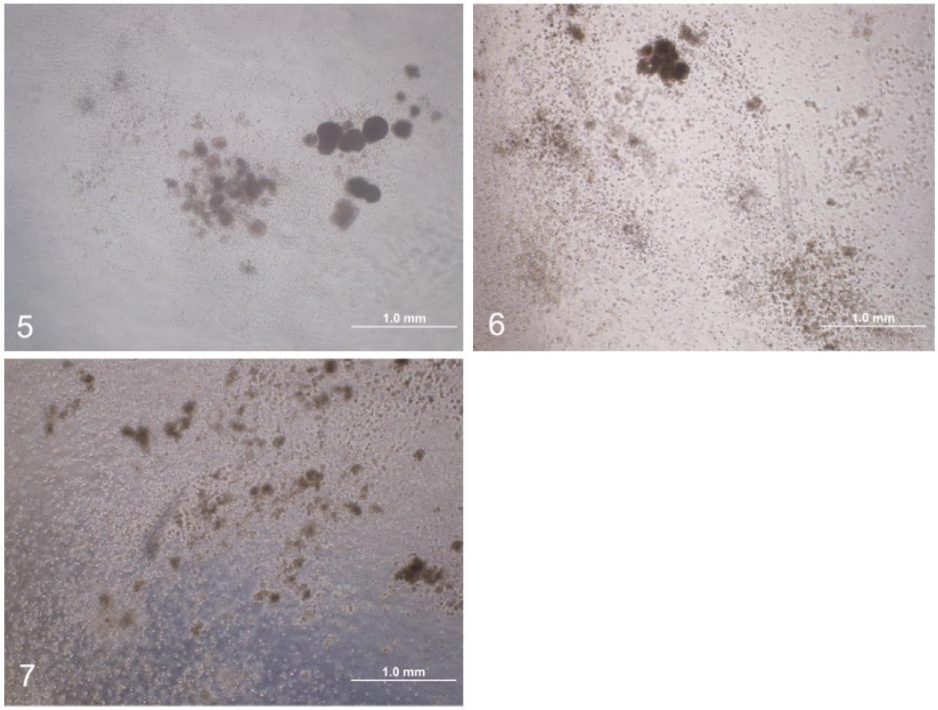

Fig. 4. CFC assays treated with dry extract of *Uncaria tomentosa*, final concentration of 200 μg/mL medium. Photo 5: CFC assay control showing the absence of overplating (40×). Photos 6 and 7: The overplating effect is demonstrated with addition of *Uncaria tomentosa* extract (40×).

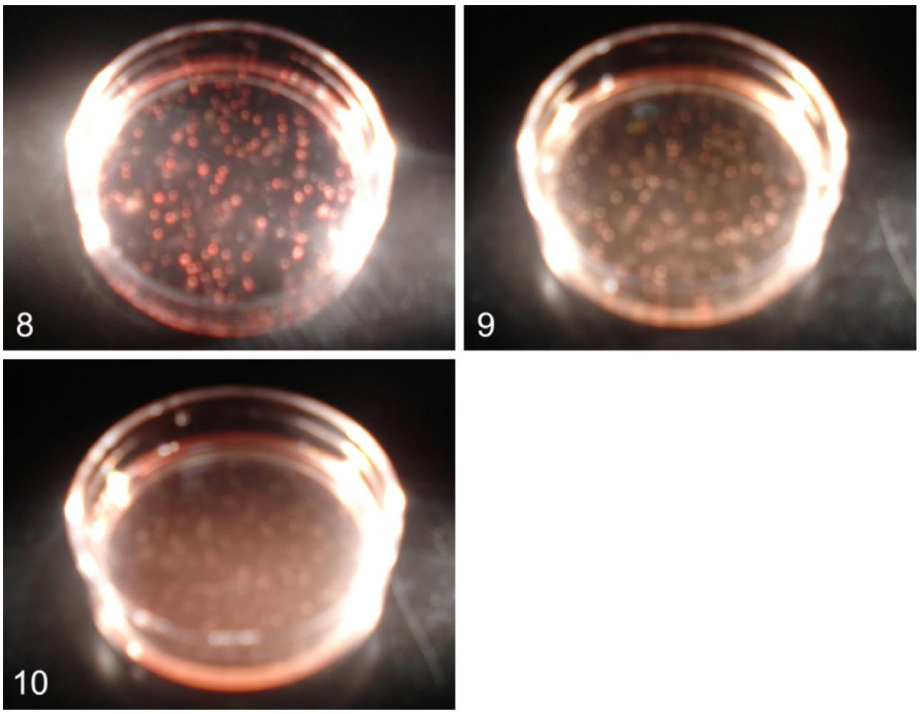

Fig. 5. Reduction in hemoglobin content in the CFU with addition of *Uncaria tomentosa* to the medium at final concentrations of 100 and 200 μg of dry extract/mL medium. Images of the plates of CFC assay control (Photo 8) and those treated with *Uncaria tomentosa* extract at final concentrations of 100 (Photo 9) and 200 (Photo 10) μg of extract/mL medium, respectively, showing the reduction of the reddish color afforded by hemoglobin (40×).

qualitative evaluation of observations in control and test culture plates.

4. Discussion

The alkaloid profile of the material used has great importance in evaluating the results obtained because POAs and tetracyclic oxindole alkaloids (TOAs) have different pharmacological properties. TOAs appear to have accentuated action in the cardiovascular and central nervous system (Shi et al., 2003); however, POAs are associated with antioxidant and anti-inflammatory action, as previously mentioned. Furthermore, TOAs are described to antagonize the pharmacological functions of POAs; therefore, inducing human endothelial cells to release a lymphocyte-proliferation-regulation factor (Wurm et al., 1998). Thus, the absence of TOAs in the sample allows its use for therapeutic and research purposes in accordance with U.S. Pharmacopeia (USP 32, 2009).

The use of ifosfamide in the present study resulted in severe neutropenia, as the control animals showed means of only 98 neutrophils/μL. Similar events are observed in clinical oncology and are a part of the focus in this study. This situation was reversed by *Uncaria tomentosa*, in which the group receiving 15 mg per animal showed 500 neutrophils/μL or higher. The administration/injection of *Uncaria tomentosa* and filgrastim during four consecutive days caused significant increases in the neutrophil count in terms of both absolute values (cells/μL) and percentage and promoted recovery from the severe ifosfamide-induced neutropenia. Even animals that received the lower dose, corresponding to 5 mg of *Uncaria tomentosa* dry extract/day, showed a significant increase in neutrophil count compared to controls. The dose tested was equivalent to 1 g/day of dry extract for a 60-kg human. Similar doses have been used for the treatment of rheumatic diseases (Castañeda et al., 1998). In this study (Castañeda et al., 1998), patients took 2 capsules of 150 mg *Uncaria tomentosa* dry extract 3 times daily for 6 months, and their hemogram, creatinine, glucose, transaminase, alkaline phosphatase, and urine were evaluated. Side effects observed include epigastric pain, dizziness, bloating, constipation, diarrhea, and bitterness in the mouth; however, all of these side effects were present only transiently. These data are consistent with those reported by National Center for Complementary and Alternative Medicine (USA) (NCCAM/NIH, 2011a), which has reported few side effects from cat's claw at the recommended dosages. Though rare, side effects may include headaches, dizziness, and vomiting; however, ambiguity exists regarding the values of "recommended dosages". The low toxicity of *Uncaria tomentosa* can be evaluated by the acute median lethal dose (LD50) to mice of an aqueous extract with 3.5% of total POA, which was found to be greater than 16 g/kg bodyweight (Keplinger et al., 1999).

The two treatments (filgrastim and *Uncaria tomentosa*) did not differ in the resulting absolute number of lymphocytes compared to controls. Sheng et al. (2000a), using an aqueous extract of *Uncaria tomentosa* (C-Med100®) in healthy rats, only observed an increase in the total number of leukocytes at doses greater than or equal to 40 mg/kg. In a subsequent study, leukopenia induced by the anticancer agent, doxorubicin, was reversed with subsequent administration of *Uncaria tomentosa*, and significant differences compared to controls at a dose of 80 mg/kg were observed (Sheng et al., 2000b). Using doxorubicin to induce leukopenia, the authors achieved a slight reduction in neutropenia.

As described from the outset, *Uncaria tomentosa* has three properties that are related to cell proliferation, particularly in terms

of WBCs: anti-inflammatory, antioxidant, and CSF-stimulating effects.

4.1. Anti-inflammatory

One of the possible pathways of action of *Uncaria tomentosa* is the prolongation of WBC survival due to the inhibited activation of NF-κB (Sandoval et al., 2000; Fazio et al., 2008), which activates several different signals and can regulate the expression of several pro-inflammatory cytokines, including TNF-α (Groom et al., 2007), interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), and interleukin-8 (IL-8). These effects subsequently decrease oxidative stress (Pilarski et al., 2006) in the cell, which in turn reduces damage to DNA (Mammone et al., 2006). This postulated effect would predict that WBCs survive longer in the circulation due to reduced DNA damage and enhanced DNA repair, thus elevating the number of immune competent cells. In the 1990s, Wurm et al. demonstrated that the POAs from *Uncaria tomentosa* stimulated endothelial cells to release a lymphocyte-proliferation-regulating factor that promoted the proliferation of normal lymphocytes and inhibited the proliferation of lymphoblasts. Akesson et al. (2003) concluded that the increase in lymphocyte counts in animals that received *Uncaria tomentosa* orally for 10 weeks was likely due to increased survival in peripheral lymphoid organs. In our study, there were no changes in the number of lymphocytes, possibly because this study took place during the acute phase.

The inhibition of NF-κB prolongs survival of WBCs; however, it is needed as a mediator for the proliferation of progenitors. Blocking the activation of NF-κB resulted in almost complete suppression of the formation of the CFU-GM, BFU-E and CFU-E progenitors. NF-κB activity is required for normal hematopoiesis and the maintenance of hematopoietic progenitor cell function, and it acts as a second messenger for a number of cytokines known to regulate hematopoiesis, such as IL-3, Epo, IL-6, SCF, and GM-CSF (Bugarski et al., 2006). Similarly, IL-6 aids granulocyte colony maintenance and the viability of mature neutrophils. IL-6 and G-CSF synergistically increase myeloid progenitors (Suzuki et al., 1996) as G-CSF facilitates the expansion of myeloid precursors, likely by stimulating the proliferation of monocytes to secrete IL-6 (Liu et al., 2005). Thus, one possible pathway for stimulating myeloid proliferation by *Uncaria tomentosa* could be the release of IL-1 and IL-6 (Lemaire et al., 1999). In the present study, the animals had a severe reduction in the number of neutrophils and monocytes. A decrease in the number of these cells impairs the production of cytokines (Groom et al., 2007) and other stimulators of the proliferation and maturation of CFU-GM. In the model of the study that we use, it is possible that the stimulation of the proliferation of myeloid tissue occurred through another pathway.

4.2. Antioxidant properties

ROS interfere with erythropoiesis because levels of reduced glutathione are directly related to mitosis. The ifosfamide used in *in vivo* tests is a DNA-alkylating agent frequently used in chemotherapy against human malignancies. Ifosfamide and its major decomposition products deplete intracellular glutathione (GSH). GSH is the major intracellular thiol reductant, protecting cells against oxidative injury. Ifosfamide depletion of intracellular GSH in human dendritic cells (DC), T cells and natural killer (NK) cells impairs their functional activity, which can be restored by reconstituting GSH (Kuppner et al., 2008). The assay of non-protein thiols was not different in animals treated with *Uncaria tomentosa*, but all groups had lower values (0.73 mmol/mL blood) than those found in the control rats of other studies (1.20 ± 0.10 mmol/mL of plasma; Spanevello et al., 2009). Also ascorbic acid, drug known to have antioxidant proprieties, did not restored non-protein thiols levels.

The use of antioxidants during cancer treatment is still controversial. This duality is true because if some antioxidants scavenge the ROS integral to the activity of certain chemotherapy drugs, which would diminish treatment efficacy, another antioxidant might mitigate toxicity and thus allow for uninterrupted treatment schedules and a reduced need for lowering chemotherapy doses (NCCAM/NIH, 2011b). Moreover, the various ROS can exert different effects according to their nature and to their intracellular level. The same phenomena occur for antioxidants, e.g., N-acetylcysteine, which decreases H_2O_2 levels, inhibits normal cell (fibroblast) proliferation but increases tumor cell proliferation (CT26 and Hepa 1–6 cells). In contrast, antioxidant molecules that mimic SOD (CuDIPS or MnTBAP) increase *in vitro* proliferation of normal cells but kill tumor cells (Laurent et al., 2005).

Even in relation to oxidative stress, levels of H_2O_2 interfere with the proliferation of myeloid precursors. Neutralization of H_2O_2 by catalase inhibits the proliferation of immature myeloid cells and also stimulates their differentiation into mature myeloid cells. SOD slightly decreases proliferation (Kusmartsev and Gabrilovich, 2003). Other studies have shown that *Uncaria tomentosa* has antioxidant activity, which is related to the reduction of tumor growth (Dreifuss et al., 2010); however, contradictory results in which a mild reduction of H_2O_2 (Gonçalves et al., 2005) and an increased activity of SOD have been observed (Pilarski et al., 2006). However, in our study, no significant differences in catalase or SOD activities were registered between treatments.

4.3. Effect on myeloid progenitors

In the case of neutropenia due to chemotherapy, when the number of WBCs is severely diminished and the scavenging activity of GSH is decreased, it is necessary to understand the route that would preferentially activate recovery from neutropenia. Therefore, to assess whether *Uncaria tomentosa* has a stimulating effect on myeloid progenitors, CFC assays were conducted using hHSPCs obtained from UCB. The addition of dry *Uncaria tomentosa* extract resulted in an increase in CFU-GM size and CFU-GEMM size at the final concentrations of 100 and 200 μg extract/mL.

It is suggested that *Uncaria tomentosa* extract may have positive effects on myeloid progenitor proliferation. These data are consistent with results obtained by Eberlin et al. (2005), who documented an increase in marrow CFU-GM and an increase in serum colony-stimulating factor CSFs in mice treated with *Uncaria tomentosa* extract (50 and 100 mg/kg).

To compare the effect on myeloid progenitors with the antiproliferative effect of neoplastic cells described above, the concentration of mitraphylline was transformed from μmol to μg/mL. Mitraphylline ($C_{21}H_{24}N_2O_4$, Molar mass 368.1736 g/mol) was used at 5–40 μmol (Garcia Prado et al., 2007; García Gimenez et al., 2010), which corresponds to 1.8–14.7 μg/mL. In the present study, 100–200 μg/mL of dry hydro-alcoholic extract containing POA 2.6–5.1 μg/mL was used, of which 0.8–1.6 μg/mL was mitraphylline. Bacher et al. (2005) used larger concentrations of four POAs (50–200 μmol); therefore, the concentration needed to stimulate the proliferation of myeloid precursors may be less than the concentration needed to induce apoptosis of neoplastic cells.A qualitative reduction in hemoglobin content was observed macroscopically and microscopically. Pilarski et al. (2009), using a chicken embryo model injected with *Uncaria tomentosa* extracts, detected unfavorable changes in Mean Corpuscular Volume (MCV), Mean Cellular Hemoglobin (MCH) and Mean Cellular Hemoglobin Concentration (MCHC). Further studies are necessary to evaluate if a toxic effect occurred and to identify the possible route of action. The leading possibility is inhibition of enzymes that participate in

the formation of heme and hemoglobin or metabolic deficiencies of Vitamin B12 and folic acid, as proposed by Pilarski et al. (2009).

5. Conclusion

At the doses tested, *Uncaria tomentosa* is promising for use in conjunction with chemotherapy because it minimizes the adverse effects of this treatment and attenuates neutropenia. More studies are needed to evaluate the interaction of *Uncaria tomentosa* with various chemotherapeutic agents and cell types.

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ARTIGO 4- Uncaria Tomentosa FOR REDUCING SIDE EFFECTS CAUSED BY CHEMOTHERAPY IN CRC PATIENTS: CLINICAL TRIAL

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Research Article

Uncaria tomentosa for Reducing Side Effects Caused by Chemotherapy in CRC Patients: Clinical Trial

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To evaluate the effectiveness of *Uncaria tomentosa* in minimizing the side effects of chemotherapy and improving the antioxidant status of colorectal cancer (CRC) patients, a randomized clinical trial was conducted. Patients (43) undergoing adjuvant/palliative chemotherapy with 5-Fluorouracil/leucovorin + oxaliplatin (FOLFOX4) were split into two groups: the UT group received chemotherapy plus 300 mg of *Uncaria tomentosa* daily and the C group received only FOLFOX4 and served as a control. Blood samples were collected before each of the 6 cycles of chemotherapy, and hemograms, oxidative stress, enzymes antioxidants, immunologic parameters, and adverse events were analyzed. The use of 300 mg of *Uncaria tomentosa* daily during 6 cycles of FOLFOX4 did not change the analyzed parameters, and no toxic effects were observed.

1. Introduction

After histological diagnosis of colorectal cancer, the treatment of advanced stages of cancer involves adjuvant or palliative chemotherapy. One common treatment plan includes the application of 5-fluoruracil (5FU)/leucovorin and oxaliplatin (FOLFOX4) [1]. However, side effects from this treatment include severe neutropenia (grade 3 or 4 according to Common Toxicity Criteria of the National Cancer Institute) in 41.1% of patients [1]. Besides the cytotoxic effect of the chemotherapy, the cause of the neutropenia may be related to oxidative stress, because high H_2O_2 levels may negatively influence the proliferation and differentiation of myeloid precursors [2]. There is a well-documented relationship between oxidative stress and colorectal cancer. Oxidative stress may result in both DNA damage [3] and the expansion of neoplastic cells, because tumoral metabolic adaptations generate high level of reactive oxygen species (ROS) [4]. Additionally, chemotherapeutic cancer treatments increase oxidative stress levels [5], resulting in high levels of reactive oxygen species (ROS) and damage to the lipids of the cytoplasmic membrane, cellular proteins, and DNA [2].

The immune status of patients, particularly $CD8^+$ and $CD4^+$ T cells (T_{regs}) levels, shows a correlation with survival. The $CD4^+/CD8^+$ ratio of the tumor infiltrating lymphocytes is significantly associated with colorectal cancer prognosis [6]. Treatment with 5FU increases IFN-γ production through the action of tumor-specific $CD8^+$ T cells that infiltrate the tumor, and it promotes T cell-dependent antitumor responses *in vivo* [7]. Again, there is an interaction between the immune response and oxidative stress. Exposure to reactive oxygen species (ROS) produced by activated granulocytes and macrophages in the context of malignant disorders causes dysfunction of T-cells and NK cells [8]. High levels of superoxide dismutase (SOD—an antioxidant enzyme) contribute to tumor cell resistance and therapy insensitivity and are correlated with poor outcome [9].

For these reasons, it is important to search for complementary treatments, including phytotherapic plants, that minimize the neutropenia associated with colon cancer chemotherapy. *Uncaria tomentosa* (*Ut*, Cat's claw) has antioxidant properties [10] and can stimulate DNA repair [11] and myelopoiesis [12]. Eberlin et al. [12] showed that *Ut* extract promotes proliferation of myeloid precursors through the increase in serum colony stimulating growth factors (CSFs). Other preclinical experiments have demonstrated the positive effect of aqueous *Ut* extract on leukocyte counts over a period of eight weeks in healthy animals [13] and after ten days of doxorubicin-induced neutropenia [14]. Given these characteristics, *Ut* could minimize the undesirable effects of chemotherapy and might improve the balance between stress and antioxidants in cancer patients. This clinical study aimed to evaluate the effect of coadjuvant treatment with *Ut* compared with conventional chemotherapy for colorectal cancer. The investigation evaluated the effect of *Ut* on oxidative stress and its consequences in relation to neutropenia, other hematological parameters, immune system, safety, and side effects.

2. Methods

2.1. Design and Patients. We performed a randomized interventional study of colorectal cancer patients who were submitted to chemotherapy treatment.

The study was carried out with 43 patients (26 female, 17 male) who had undergone complete resection of their colorectal cancer, which was of histologically scored as stage IIB, III, or IV, and who were going to begin adjuvant/palliative chemotherapy with FOLFOX4 at the *Hospital Universitário de Santa Maria*, Brazil.

Patients were randomly grouped into two groups, according to the date of treatment start as follows: the first patient who agreed participating in the study was included into UT group, the second into the C group, and successively until the end. The UT group was treated with FOLFOX4 plus *Ut*, and the control group (C) received only FOLFOX4. Patients remained on study during 6 chemotherapies cycles, of 15 days each. The doses of medication in UT group were as follows: Oxaliplatin, 85 mg/m^2 on day1; 5FU, 1 g/m^2 on days 1 and 2; Leucovorin, 200 mg/m^2 on days 1 and 2; and *Ut* (Unha de Gato Herbarium), 3 tablets daily from day 3 to day 15. The dose of *Ut* is similar to that used in previous study, using 250–350 mg C-MED-100, an aqueous extracts of *Ut* [11]. No changes in food intake pattern had occurred during the supplementation.

The calculation for estimating the sample size required for a randomized clinical trial was according to Greenberg et al. [15], with constant level of significance (α) of 5%, and statistical power of 90% (β 10%), using as a reference the study of Sheng et al. [13].

The Human Ethics Committee of the *Universidade Federal de Santa Maria* approved this study, and informed consent was obtained from all participants (protocol n. 0169.0.243.000-07).

2.2. Materials. Each tablet of Unha de Gato Herbarium contained 100 mg dry *Ut* extract. Biological materials used in the tablets were derived from plants in their natural habitat. The *Ut* extract was prepared by ultra-turrax extraction (Biotron, Kinematica AG) from ground bark (Centroflora) using 70% ethanol (Dipalcool). The HPLC analysis of *Ut* dry extract presents the content of 2.57% pentacyclic oxindole alkaloids (POAs), which were calculated with reference to external calibration curves of mitraphylline. The analysis of extract showed absence of tetracyclic oxindole alkaloids in the sample, allowing its use for therapeutic and research purposes in accordance with U.S. Pharmacopeia.

2.3. Sample Collection. Blood was collected in citrated, EDTA, heparin, and without anticoagulant Vacutainer tubes, before chemotherapy and after each of the 6 cycles. CAT and SOD activities were determined using whole blood diluted in a 1 : 20 saline solution.

2.4. Biochemical Parameters. A COBAS INTEGRA system was used for the quantitative determination of the chemical constituents of the blood, and data were acquired using a COBAS INTEGRA 400 plus apparatus (USA).

2.5. Carbonylation of Serum Protein. The carbonylation of serum proteins was determined through a modification of the Levine method [16].

2.6. Determination of Lipid Peroxidation. Lipid peroxidation was estimated by measuring TBARS in plasma samples according to a modification of the method of Jentzsch et al. [17].

2.7. Catalase (CAT) and Superoxide Dismutase (SOD) Activities. The determination of CAT activity level was carried out in accordance with a modification of the method of Nelson and Kiesow [18]. SOD activity was calculated based on the ability to inhibit the reaction of superoxide and adrenaline, as described by McCord and Fridovich [19].

2.8. Hemograms. Blood samples were analyzed using a Pentra apparatus (France). The lowest values were confirmed by observation of slides using May Grünwald-Giemsa stain and optical microscopy.

2.9. Interleukin 6 (IL-6). ELISA assays of IL-6 were carried out according to a previously published method [20] at room temperature in 96-well microtitre plates (Nunc-Immuno Plate Maxi Sorp), and optical densities (O.D.) at 490 nm were determined using a microplate reader (Thermo Scientific Multiskan FC, Vantaa, Finland).

2.10. Single Cell Gel Electrophoresis (Comet Assay). Alkaline comet assays were performed as described by Singh et al. [21]. One hundred cells (50 cells from each of the two

replicate slides) were selected and analyzed. The slides were analyzed under blind conditions by at least two different individuals.

2.11. CD3$^+$, CD4$^+$, and CD8$^+$ Cells. Samples were collected in EDTA, and analyses were performed using a three-color fluorescence-activated cell sorter (FACScalibur, Becton Dickinson Biosciences, USA) and Multiset software (Becton Dickinson). FITC-conjugated anti-CD4, PE conjugated anti-CD8 and PerCp conjugated anti-CD3 were used. Immune subpopulations were measured as a percentage of the total number of CD3$^+$ cells.

2.12. Adverse Events. The Common Terminology Criteria for Adverse Events (AE) v3.0 (CTCAE) from the National Institutes of Health/National Cancer Institute-EUA [22] has been used. Grade refers to the severity of the AE. The CTCAE v3.0 employs grades from 1 to 5, with unique clinical descriptions of severity for each AE based on the following general guideline: grade 1, mild AE, grade 2, moderate AE, grade 3, severe AE, grade 4, life-threatening or disabling AE, and grade 5, death related to an AE. The adverse events were judged in terms of clinical symptoms by interview at each chemotherapy cycle with pharmaceutical. Serum clinical chemistry, whole blood analysis, and leukocyte differential counts were also used to monitor the efficacy and toxicity by physicians of the Department of Oncology.

2.13. Statistics. Data were analyzed using the EpiInfo computer program, version 3.5.1 from the CDC, USA. The data were evaluated using analysis of variance (ANOVA) and t-test and were expressed as a mean ± SD. When the variances were not homogenous and ANOVA was not appropriate (Bartlett's P value < 0.05), Wilcoxon two-sample test was used to evaluate the data. $P < 0.05$ was considered statistically significant.

3. Results

The general characteristics of colorectal cancer patients included in this study are shown in Table 1. The mean age of the C group was 60.89 years, and the mean age of the UT group was 62.68 years old.

One aim of the study was to evaluate the neutropenia, thrombocytopenia, and anemia. The hemograms were analyzed each 15 days, and there were no significant differences in hematological parameters (Tables 2 and 3) between the groups for any of the cycles examined. An important reduction in white blood cells (WBCs) count was observed in both groups along the treatment. Unlike what was observed in leukocytes, red blood cells (RBCs) showed recovery of the hypochromic and microcytosis present at baseline. Erythrocyte indices (mean corpuscular hemoglobin-MCH; mean corpuscular volume, MCV) improved in both groups, reaching normal values.

The generation of ROS may damage all types of biological molecules. Oxidative damages to lipids, proteins, or DNA were evaluated by TBARS levels, protein carbonyl levels, and comet assay, respectively. Antioxidant defense system was measured by activity of antioxidants enzymes catalase and SOD. The *Ut* supplementation did not change oxidative stress values or activity of the antioxidant enzymes. Similarly, the comet assay, a sensitive technique for the detection of DNA damage at the level of the individual eukaryotic cell, demonstrated no differences between groups (Table 4).

Table 1: General data of the CRC patients in adjuvant/palliative chemotherapy (FOLFOX4) without *Uncaria tomentosa* supply (C group) or receiving 300 mg/day of *Uncaria tomentosa* (UT group).

Parameters		*C group* $n = 23$	*UT group* $n = 20$
[a]States T	T1	0	1
	T2	1	1
	T3	16	12
	T4	6	6
[a]States N	N0	1	2
	N1	8	8
	N2	11	10
[a]States M	M0	13	17
	M1	10	3
Age	<50	4	2
	51–65	10	12
	66–80	9	6
Gender	Female	16	10
	Male	7	10
Smoke	Smokers	2	0
	ex-smokers	8	10
	no smokers	13	10
Associated chronic diseases	Without	5	4
	Hypertension	6	6
	Hypertension + coronary diseases/dyslipidemia/diabetes	3	7
	Diabetes	2	2
	Others	4	1

[a] Stage of disease currently described by TNM, as published by the American Joint Committee on Cancer (AJCC) and American Cancer Society (ACS).

The CD4$^+$ T cells and CD8$^+$ T cells (absolute count and ratio) used for the evaluation of the immune status of CRC patients were not statistically different following *Ut* supplementation (Table 5) or chemotherapy (before treatment began versus after the sixth FOLFOX4 cycle). The UT and C groups showed differences before chemotherapy in IL-6 levels, but such differences did not change over the course of treatment. Moreover, in the comet assays, IL-6 levels showed large variations between subjects.

The assessment of adverse events (AEs) related to treatment was conducted by interviewing the patients during each cycle of chemotherapy and through analysis of laboratory tests and observation of abnormal symptoms presented by patients. AEs were classified according to the Common Terminology Criteria for Adverse Events v3.0 (CTCAE v3.0) [22]. Since there were no differences between groups, data

Table 2: Evaluation of hemoglobin, and erythrocyte indices of the CRC patients, during six cycles of adjuvant/palliative chemotherapy, without *Uncaria tomentosa* supply (C group) or receiving 300 mg/day of *Uncaria tomentosa* (UT group).

Parameters	*Group*	*Chemotherapy cycle*						
		0	1	2	3	4	5	6
Hb	UT	123.5	125.2	127.2	123.5	125.3	121.4	118.6
g/L		(14.9)	(17.9)	(17.0)	(14.2)	(13.9)	(11.1)	(10.4)
	C	113.7	115.1	113.3	113.9	114.9	117.0	113.7
		(17.6)	(13.8)	(15.7)	(17.2)	(14.2)	(12.5)	(14.2)
MCH	UT	27.53	27.88	28.37	28.95	29.66	29.69	30.60*
pg		(1.99)	(1.69)	(1.97)	(2.20)	(2.31)	(1.92)	(2.16)
	C	26.62	27.02	27.37	27.70	27.93	28.80	29.19*
		(2.61)	(2.63)	(2.59)	(2.67)	(2.63)	(2.60)	(2.44)
MCV	UT	84.62	85.31	86.03	87.57	89.13	90.00	91.50**
fL		(4.31)	(3.91)	(3.65)	(5.36)	(5.59)	(4.90)	(4.84)
	C	81.94	82.53	83.74	85.28	86.27	88.29	89.22**
		(6.8)	(6.58)	(6.4)	(6.34)	(6.02)	(5.92)	(5.51)

Values expressed as mean (SD). Hb: hemoglobin, MCH: mean corpuscular hemoglobin, MCV: mean corpuscular volume. UT group: patients treated with FOLFOX4 + *Uncaria tomentosa* 300 mg/day ($n = 20$), C group: patients treated with FOLFOX4 ($n = 23$); $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$ in relation to the day 0.

Table 3: White blood cells count and platelets's evaluation of the CRC patients in adjuvant/palliative chemotherapy, without *Uncaria tomentosa* supply (C group) or receiving 300 mg/day of *Uncaria tomentosa* (UT group).

Parameters	*Groups*	*Chemotherapy cycles*						
		0	1	2	3	4	5	6
WBC	UT	8.178	5.689*	5.184**	4.778**	4.956**	4.660**	4.442**
cells $\times 10^9$/L		(2.705)	(1.919)	(2.051)	(2.022)	(2.006)	(2.095)	(2.433)
	C	7.668	5.933*	5.391*	4.886*	4.473***	4.962**	4.195***
		(2.892)	(2.137)	(2.451)	(2.039)	(2.086)	(2.157)	(1.558)
Neutrophils	UT	5.345	3.069*	2.600**	2.441**	2.429**	2.227**	2.343***
cells $\times 10^9$/L		(2.691)	(1.455)	(1.411)	(1.401)	(1.456)	(1.506)	(1.842)
	C	4.871	3.285*	2.848**	2.295***	2.190***	2.514*	1.975***
		(2.674)	(1.618)	(1.858)	(2.295)	(1.628)	(1.797)	(1.186)
Lymphocytes	UT	2.038	1.888	1.859	1.673	1.776	1.728	1.433*
cells $\times 10^9$/L		(.735)	(.681)	(.656)	(.642)	(.661)	(.754)	(.504)
	C	1.901	1.888	1.815	1.769	1.650	1.697	1.578
		(.702)	(.737)	(.717)	(.827)	(.648)	(.531)	(.659)
Monocytes	UT	.538	.515	.528	.482	.546	.547	.542
cells $\times 10^9$/L		(.215)	(.157)	(.197)	(.216)	(.244)	(.241)	(.306)
	C	.542	.533	.570	.602	.470	.595	.498
		(.245)	(.254)	(.254)	(.272)	(.197)	(.262)	(.231)
Platelets	UT	263	188*	174*	146***	135***	117***	117***
count $\times 10^9$/L		(92)	(43)	(55)	(48)	(51)	(42)	(33)
	C	286	216*	197**	161***	149***	165***	141***
		(76)	(68)	(64)	(70)	(66)	(57)	(58)

Values expressed as mean (SD). UT group: patients treated with FOLFOX4 + *Uncaria tomentosa* 300 mg/daily ($n = 20$); C group: CRC patients received FOLFOX4 ($n = 23$); $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$ in relation to the day 0.

shows that *Ut* supplementation did not alter the occurrence of AEs, related to chemotherapy, neither caused AE. The most frequently observed AE in both groups were fatigue, nausea, and a decrease in hematological parameters (Tables 6 and 7). An important reduction in neutrophils (grade 3 or 4) occurred in 25.4% of patients. Toxicity of the *Ut* was also evaluated using liver, kidney, metabolic, and constitutive parameters. Treatment with *Ut* did not alter liver function, defined as elevation of liver enzymes (alanine aminotransferase-ALT, aspartate aminotransferase-AST, γ

Table 4: Evaluation of lipid peroxidation, carbonylation of serum protein, DNA damage and antioxidant defenses of the CRC patients in adjuvant/palliative chemotherapy (FOLFOX4), without *Uncaria tomentosa* supply (C group) or receiving 300 mg/day of *Uncaria tomentosa* (UT group).

Parameters	*Group*	*Chemotherapy cycles*						
		0	1	2	3	4	5	6
TBARS nmol	UT	16.7	16.2	18.3	17.9	17.7	17.8	21.6
MDA/mL		(9.34)	(7.1)	(7.2)	(6.9)	(4.1)	(3.4)	(11.8)
	C	22.5	18.8	17.8	19.4	21.2	22.4	22.9
		(11.6)	(10.9)	(11.3)	(10.5)	(8.9)	(9.7)	(10.5)
Protein carbonyl	UT	0.63	0.56	0.61	0.61	0.68	0.67	0.6
nmol/mg protein		(0.2)	(0.2)	(0.17)	(0.18)	(0.26)	(0.2)	(0.2)
	C	0.79	0.77	0.73	0.72	0.78	0.9	0.84
		(0.37)	(0.4)	(0.39)	(0.36)	(0.37)	(0.43)	(0.37)
Comet assay	UT	29.04						26.78
Index damage		(34.18)						(31.99)
	C	26.94						34.66
		(49.3)						(46.63)
Catalase	UT	7.85	8.2	7.65	9.12	8.97	9.33	10.39
pmol/mg protein		(3.3)	(3.0)	(2.81)	(4.75)	(4.71)	(3.27)	(4.08)
	C	9.05	8.29	8.51	8.06	9.07	8.31	9.38
		(4.9)	(2.7)	(4.05)	(3.51)	(3.71)	(3.07)	(3.78)
SOD	UT	1.82	1.85	1.95	2.19	1.95	2.29*	2.41*
U/mg protein		(0.5)	(0.44)	(0.44)	(0.43)	(0.43)	(0.44)	(0.56)
	C	1.91	1.91	2.1	2.15	2.09	2.12	2.13
		(0.8)	(0.76)	(0.71)	(0.78)	(0.7)	(0.81)	(0.72)

Data expressed in mean (SD). TBARS, thiobarbituric acid-reactive substances; SOD, superoxide dismutase; UT group: patients treated with FOLFOX4 + *Uncaria tomentosa* 300 mg/daily ($n = 20$); C group: CRC patients received FOLFOX4 ($n = 23$). $^*P < 0.05$ in relation to the day 0.

Table 5: Immune status of CRC patients before treatment began and after 6 cycles of adjuvant/palliative chemotherapy (FOLFOX4) without *Uncaria tomentosa* supply (C group) or receiving 300 mg/day of *Uncaria tomentosa* (UT group).

Parameters	*Group*	*Chemotherapy cycles*	
		0	6
$CD4^+$ T Cells	UT	958.36 (414.6)	720.28 (271.72)
Cells/μL	C	828.45 (431.25)	848.38 (430.56)
$CD8^+$ T cells	UT	494.0 (231.28)	390.64 (223.62)
Cells/μL	C	490.29 (271.14)	394.27 (149.96)
$CD4^+$ T/$CD8^+$ T Ratio	UT	2.17 (0.76)	2.21 (0.78)
	C	1.96 (1.03)	2.30 (1.08)
IL6	UT	$4.07^{\#}$ (6.54)	$5.1^{\#}$ (6.12)
ng/mL	C	12.97 (13.28)	16.66 (18.2)

Data expressed as mean (SD); UT group: patients treated with FOLFOX4 + *Uncaria tomentosa* 300 mg/daily ($n = 20$); C group: CRC patients received FOLFOX4 ($n = 23$). $^{\#}P < 0.05$ between groups.

glutamyl transpeptidase-GGT), and bilirubin levels, and kidney function is evaluated by dosage of urea, metabolic parameters (albumin levels and glycemia), and constitutive parameters (weight loss) (data not shown). There was a small difference in creatinine levels between groups before treatment (UT = 74.25 μmol/L, C = 68.95 μmol/L), which remained at the sixth cycle of treatment (UT = 76.90 μmol/L, C = 60.12 μmol/L).

4. Discussion

Complementary and alternative medicine (CAM) has been used by a large number of cancer patients worldwide. Cultural, socioeconomic, and spiritual differences affect the rate of use. For instance, there are high rates of use in Mexico (97.2%) and China (97%) [23, 24], an intermediate rate of use in the USA (63%) [25], and lower rates of use in Canada (47%) [26] and Iran (35%) [27]. The herbal remedy

Table 6: Frequency of side effects reported by CRC patients in adjuvant/palliative chemotherapy in interview at first and sixth cycle of the treatment.

	After 1 chemotherapy cycle					*After 6 chemotherapy cycles*				
	Not present	rarely	sometimes	often	Always	Not present	Rarely	Sometimes	Often	always
Fatigue	60.5	13.2	13.2	5.3	7.9	54.3	5.7	22.9	5.7	11.4
Insomnia	86.8	2.6	7.9	2.6	0	97.1	2.9	0	0	0
Vomiting	78.9	10.5	10.5	0	0	100	0	0	0	0
nausea	60.5	15.8	21.1	2.6	0	80.0	15.0	0	0	5.0
Dry skin	91.2	2.9	2.9	2.9	0	88.6	0	0	5.7	5.7
Pruritus/itching	81.6	2.6	10.5	0	5.3	91.4	5.7	0	0	2.9
Fever	97.4	2.6	0	0	0	97.1	2.9	0	0	0

Data expressed as % of the patients who had symptoms.

Table 7: Adverse events observed in CRC patients in adjuvant/palliative chemotherapy with FOLFOX4 ($n = 43$).

	After 1 chemotherapy cycle (FOLFOX4) Grade refers to the severity of the AE*					*After 6 chemotherapy cycles (FOLFOX4)* Grade refers to the severity of the AE				
	0	1	2	3	4	0	1	2	3	4
Hemoglobin	82.9	12.2	4.9	0	0	76.9	17.9	2.6	0	0
Leukocytes (total WBC)	97.6	2.4	0	0	0	74.4	5.1	15.4	5.1	0
Neutrophils/granulocytes	85.4	7.3	0	4.9	2.4	48.7	12.8	10.3	17.9	7.7
Lymphocytes	95.1	0	4.9	0	0	92.3	0	7.7	0	0
Platelets	97.6	0	2.4	0	0	84.6	10.3	5.1	0	0
Weight loss	82.9	9.8	2.4	4.9	0	92.3	0	2.6	5.1	0
Hyperglycemia	97.6	0	0	2.4	0	87.2	0	7.7	5.1	0
γ-glutamyl transpeptidase	100	0	0	0	0	89.8	10.2	0	0	0
Alkaline phosphatase	100	0	0	0	0	84.6	15.4	0	0	0
Neuropathy: sensory	100	0	0	0	0	94,9	0	5.1	0	0
Infection with Grade 3 or 4 neutrophils	100	0	0	0	0	97.4	0	2.6	0	0
Diarrhea	100	x	0	0	0	92.3	x	7.7	0	0

Values expressed as % of the patients who had AE. *Grade refers to the severity of the AE: grade 1 = mild AE; grade 2 = moderate AE; grade 3 = severe AE; grade 4 = life-threatening or disabling AE; grade 5 = death related to an AE [22].

cat's claw is used as CAM by some cancer patients. It is thought to be an anticarcinogen, an immunostimulant, and an antioxidant that can stimulate DNA repair. Despite a theoretical understanding of its mechanism of action, the evidence for its clinical effectiveness is minimal [28].

To evaluate the effectiveness of *Ut* in minimizing the main side effects of chemotherapy, a decrease in neutrophil and platelet counts, hemograms were analyzed before each FOLFOX4 cycle (at an interval of 15 days). Treatment with *Ut* was suspended on the days that patients received chemotherapy, because some antineoplastic drugs and CAMs are metabolized through the cytochrome P450 family, and an interaction could alter the metabolism of patients [28]. Treatment with *Ut* did not improve the WBC, RBC, or platelet counts, because there were no differences between groups (UT versus C). *In vivo* assays in rats undergoing chemotherapy demonstrated that neutrophils recover significantly sooner with *Ut* supplementation [14]. This effect of *Ut* on WBC is due both to the stimulation of myeloid precursors [12] and the effect on ROS, which increases survival of lymphocytes [29] and inhibits myeloid cell differentiation [2]. We did not find other clinical trials with CRC patients using *Ut*. In a human volunteer study, *Ut* extract was given at 250 or 350 mg/day for 8 consecutive weeks to healthy adult. There were no statistically significant differences among the groups in WBC [11]. Our group conducted a clinical trial of women with breast cancer undergoing chemotherapy treatment who received 300 mg of *Ut* daily. In this study, we found significant differences between the group that received *Ut* and the control group; the *Ut* group showed higher neutrophils counts compared with the control group (unpublished data). The differences in the drugs used in the treatment of breast cancer (5-FU, adriamycin, and cyclophosphamide) versus CRC (5-FU and oxaliplatin) have to be considered, as do the differences in time between cycles of chemotherapy (21 versus 15 days). We must also consider the fact that all CRC patients in the present study underwent colectomy, which could interfere with the absorption of *Ut*.

Many previous studies have clearly shown the potential of the antioxidant *Ut*, and its potent radical scavenger activity was confirmed by several assays including the following: the

capacity to reduce the free radical diphenylpycrilhydrazyl (DPPH assay) [30, 31], the reaction with the superoxide anion, peroxyl [30], and hydroxyl radicals [30] as well as with the oxidant species, hydrogen peroxide, and hypochlorous acid [30, 32], and the TEAC assay [10]. The antioxidant activity of *Ut* extracts was further assayed through determination of TBARS production (using rat liver homogenates and sarcoplasmic reticulum membranes) and by the inhibition of free radical-mediated DNA-sugar damage [30, 33]. These assays were primarily *in vitro* tests with one *in vivo* test [31]. Despite this strong evidence, no differences in oxidative stress were found between groups that received or did not receive *Ut*, as assessed by lipid peroxidation (TBARS) and protein carbonyls. In addition, no differences were observed in the antioxidant enzymes SOD or catalase.

There are close correlations between DNA damage, DNA repair, and immune responses in lymphocytes. DNA damage and mutations may result in a failure of T cells to proliferate and undergo extensive clonal expansion upon antigenic stimulation. Sheng et al. [11] showed that a water-soluble extract of *Ut* caused a significant decrease in DNA damage and a concomitant increase in DNA repair in volunteers. However, in our study, the comet assay did not demonstrate a significant difference in the group that received *Ut*. *Ut* extract was prepared through an extraction of ground bark with 70% ethanol. This process altered the composition of the extract (oxindole alkaloids) compared with an aqueous extract (like that used by Sheng et al. [11]). However, more recently, water-soluble cat's claw extract was shown not to contain significant amounts of alkaloids (<0.05%). Yet, it was still shown to be efficacious, because quinic acid is the major active ingredient [34]. Further study is needed to assess whether differences in the content of the extracts are correlated with the differences in observed results.

Similar to oxidative stress, *Ut* did not show an effect on the analyzed immunologic parameters, the $CD4^+$ T cell-$CD8^+$ T cell count and the IL-6 levels despite the *in vitro* evidence [35].

There were no drug-related toxic effects observed for *Ut* extract at a repeated dose of 300 mg/day for 12 consecutive weeks (Unha de Gato Herbarium), when judged in terms of clinical symptoms, serum clinical chemistry, whole blood analysis, and leukocyte differential counts. Similar results have been shown in previous studies with volunteers [11, 36]. National Center for Complementary and Alternative Medicine (USA) [37] has reported few side effects from cat's claw at the recommended dosages. Though rare, side effects may include headaches, dizziness, and vomiting.

Adverse events related to antineoplastic drugs (oxaliplatin and 5FU) are well known [1] and are similar to those observed in our study.

5. Conclusion

Ut at dose 300 mg dry extract daily is not effective in reducing the most prevalent adverse events due to treatment with 5FU/Leucovorin and oxaliplatin in patients with advanced CRC. No toxic effects related to *Ut* were observed in the group that received 300 mg dry extract daily for 12 weeks. Additional studies are needed to evaluate under which conditions, drugs, or types of cancer *Ut* might have a positive effect on treatment, in decreasing neutropenia and thrombocytopenia, or in improving the immune response.

Conflict of Interests

All the authors deny any conflict of interests. This work had a financial support from the government agencies CNPq and CAPES.

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DISCUSSÃO

Há uma clara relação entre os estágios do câncer cólon retal, e em consequência com o prognóstico, e a densidade de linfócitos T infiltrantes no tumor, com os estágios avançados relacionados com menores valores de células T citotóxicas ($CD8^+$)(SALAMA et al., 2009). Assim como os linfócitos $CD8^+$, os linfócitos auxiliares ($CD4^+$) apresentam propriedades antitumorais, enquanto os linfócitos reguladores ($CD4^+$ $CD45^+FOX3^+$) (T_{reg}) suprimem essas propriedades (WALDNER et al., 2006). Com relação às células T $CD4^+$, há algumas contradições em relação ao sub-grupo T_{reg}. Apesar da forte capacidade de inibir a atividade das células CD8, as células T_{reg} foram encontradas em menor quantidade nos tumores de maior estágio T, sendo relacionadas ao melhor prognóstico quando em menor número nos tecidos normais adjacentes ao tumor (SALAMA et al., 2009). Possivelmente, sua atividade supressora da atividade citolítica dos linfócitos $CD8^+$ seja mais efetiva nos tecidos adjacentes, interferindo nas fases de equilíbrio e escape (KOEBEL et al., 2007). No presente estudo, avaliando a fenotipagem dos linfócitos do sangue periférico, encontramos contagens menores de linfócitos $CD8^+$ nas mulheres em relação aos homens. Essas diferenças não foram significativas quando avaliada em relação aos estadios TNM, possivelmente devido à homogeneidade de nossa amostra, constituída na sua maioria por pacientes com câncer em estadios avançados. Pacientes com menores valores de linfócitos $CD8^+$ apresentaram maior risco de progressão da doença (grupo PO)(RR= 2,44). Um mecanismo, pelo qual os linfócitos T infiltrantes do tumor suprimem a proliferação das células tumorais, ocorre via inibição da produção de IL6 (BECKER et al., 2005). Os níveis de IL6 foram maiores no grupo PO, apesar de já ter ocorrido a remoção do tumor (colectomia) e aumentaram a despeito do tratamento com a quimioterapia (esquema FOLFOX4), o que indica que os linfócitos T não foram efetivos em inibir a produção de IL6. Outro dado observado foi a forte correlação dos níveis de hemoglobina e a contagem de linfócitos $CD8^+$. Uma possível via relacionada à essa observação refere-se ao fato da anemia ser um dos fatores associados com o aporte de oxigênio ao tecido tumoral. A hipóxia resulta na estabilização do HIF1α e no acúmulo de adenosina extracelular, e assim consequente elevação de cAMP intracelular (SITKOVSKY, 2008). O ATP, no espaço extracelular, é citotóxico para certos tumores, sendo então degradado à adenosina pelas ectoenzimas NTPDase (ecto-apirase, CD39) e 5'-nucleotidase (CD73). A adenosina é degradada pela ectoenzima adenosina deaminase (ADA), que se liga a

superfície celular através do CD26. Porém, a adenosina pode inibir a transcrição e expressão do CD26 nas células de carcinoma colorectal (HT29). O resultado é uma alta concentração de adenosina no ambiente tumoral, o que suprime a ativação de linfócitos T, mediada pela falha na apresentação de antígeno ao receptor de células T (TCR), através do complexo de histocompatibilidade principal (MHC), em parte por sinalização através do receptor de adenosina A_{2A} (LINDEN, 2006). Esta inibição adenosinérgica pela hipóxia, através dos receptores A_{2A} na superfície dos linfócitos $CD8^+$, resulta em forte inibição da atividade antitumoral (SITKOVSKY, 2008).

A hipóxia também está relacionada à geração de EROS e, como citado anteriormente, promovendo a estabilização do HIF1α. A estabilização do HIF1α é o fator desencadeante de uma série de eventos mediados por vários oncogenes (Ras, Src, Bcl-Abl)(PELICANO et al., 2004), que ocasionam trocas na expressão gênica de várias enzimas (hexokinase2, TKTL1, PDK) (ROUDIER e PERRIN, 2009), que resultam na adaptação metabólica da célula tumoral, favorecendo o surgimento e desenvolvimento do câncer. As enzimas lactato desidrogenase (LDH), gama glutamil transpeptidase (GGT), fosfatase alcalina (FAL) junto com o antígeno carcinoembrionário (CEA) são comumente usadas na clínica médica como biomarcadores de câncer cólon retal. Dessas enzimas, sabe-se que a LDH é regulada positivamente pelo HIF1α e o aumento de sua atividade torna eficiente o metabolismo anaeróbico/glicolítico das células tumorais, por regenerar NAD^+ rapidamente e em quantidade suficiente (KOUKOURAKIS et al., 2006). A análise dos dados do presente estudo demonstra que há uma forte correlação dos níveis de TBARS com essas enzimas relacionadas à adaptação metabólica das células tumorais, demonstrando que o estresse oxidativo tem um papel central nesse processo. De forma semelhante, ocorre correlação positiva com os níveis de CEA. Os pacientes do grupo PO apresentaram níveis elevados de todos os biomarcadores, em relação aos pacientes do grupo GO e aos indivíduos saudáveis. No grupo em que ocorreu a progressão da doença (grupo PO), ocorreu elevação dos biomarcadores na vigência da quimioterapia, mas a elevação dos níveis de TBARS ocorreu de maneira precoce, a partir do terceiro ciclo de quimioterapia, ou seja, após 45 dias do início do tratamento. Esse dado é valioso, pois nesse período ainda não são previstos exames de imagem, como tomografia computadorizada e ultrassonografia, que permitiriam identificar o surgimento de novas metástases ou mesmo aumento nos implantes prévios, indicando a falha na resposta terapêutica.

Ainda em relação ao estresse oxidativo, os níveis de EROs, em especial os níveis de H_2O_2 desempenham um papel fundamental na indução da apoptose. Portanto as enzimas

antioxidantes, como a Mn-SOD, que diretamente interferem nos níveis desses elementos, são cruciais tanto na apoptose quanto na resistência das células tumorais à quimioterapia. Os maiores níveis Mn-SOD em carcinomas de colón retal contribuem para a resistência das células tumorais e insensibilidade à terapia, resultando em um pior prognóstico (JANSSEN et al., 1998). Os níveis de antioxidantes, como da enzima SOD, não refletem necessariamente o estado redox de uma célula. Altos níveis de antioxidantes celulares comumente ocorrem nas células submetidos ao estresse oxidativo. Em nosso estudo, observou-se um aumento na atividade da SOD dos pacientes com câncer colon retal em relação ao grupo controle. De forma aparentemente contraditória, foi observada discreta elevação nas enzimas antioxidantes Catalase e SOD após seis ciclos de quimioterapia no grupo GO. No entanto, Hussain et al. (2004) demonstraram que a apoptose induzida pelo gene p-53, em linfoblastos expostos à doxorubicina, era mediada pelo aumento da expressão da Mn-SOD. As células com p-53 mutante, não funcional, não demonstravam elevação da Mn-SOD e, em consequência, apresentavam resistência ao quimioterápico. Priego et al. (2008) confirmaram o papel central da Mn-SOD na eliminação das células de carcinoma de cólon (HT29) em animais tratados com esquema FOLFOX. Muitos pacientes com câncer colón retal apresentam mutações no gene p-53, relacionadas com uma pobre resposta terapêutica. Uma análise por biologia molecular do gene p-53 dos pacientes em estudo, poderia melhor esclarecer se o aumento da SOD no grupo GO estava relacionado à forma "não mutante" do gene p-53.

O principal efeito colateral do esquema FOLFOX4 para tratamento do câncer cólon retal refere-se à toxicidade medular, sendo a neutropenia o mais prevalente evento adverso. No presente estudo, a neutropenia estava presente em 51,3% dos pacientes em tratamento. Quando a contagem de neutrófilos é inferior a 500 cel/mm³ (grau 4 conforme *Common Terminology Criteria for Adverse Events*, NATIONAL CANCER INSTITUTE, 2009) o tratamento deve ser suspenso, podendo comprometer a resposta terapêutica.

O uso de extrato hidroalcoolico de *Uncaria tomentosa*, em modelo animal, resulta em aumento dos precursores de granulócitos e macrófagos (CFU_GM) na medula óssea e aumento sérico dos fatores estimuladores de colônias (CSFs) (EBERLIM et al., 2005). Para avaliar a capacidade da *Uncaria tomentosa* em recuperar a neutropenia causada por quimioterapia, foi realizado ensaio biológico com camundongos, usando ifosfamida para ablação medular e filgrastim como droga referência. O extrato de *Uncaria tomentosa*, nas doses de 5 e 15 mg/dia, via oral causou um importante aumento no número de neutrófilos, com potência equivalente à 85,2% da droga referência, filgrastim, nas dose de 3 e 9 µg/dia, via intraperitoneal, respectivamente. O tratamento com *Uncaria tomentosa*, assim como com

filgratim, não alterou o número de linfócitos, sugerindo um efeito sobre os precursores mielóides. Já o tratamento com ácido ascórbico, usado como antioxidante padrão, causou importante aumento no número de linfócitos e apenas discreta elevação nos neutrófilos. Esses dados são compatíveis com os estudos de Sheng et al. (2000a) e Akesson et al. (2003; 2005), que usaram um extrato aquoso, que apresenta uma maior concentração do ácido quínico e praticamente concentração nula de alcalóides oxindólicos, e que resultou em aumento do número de linfócitos em modelo animal. Assim, os extratos aquosos possuem maior relação com as propriedades imunomoduladoras, mediadas por citocinas como TNFα (SANDOVAL et al., 2002), de reparo celular devido à exposição física ou química (GUTHRIE et al., 2011; SHENG et al., 2001), e, em maiores concentrações, com as atividades antioxidantes (SANDOVAL et al., 2002) e os extratos alcoólicos e/ou os alcaloides oxindólicos pentacíclicos isolados, aos efeitos mieloproliferativos e antiproliferativos de células tumorais (BACHER et al., 2005; GARCIA GIMENEZ et al., 2010; GARCIA PRADO et al., 2007; PILARSKI et al., 2010). Ainda em relação às atividades antioxidantes, os extratos etanólicos também apresentam propriedades antioxidantes (PILARSKI et al., 2006), apesar de ainda ser controversa a natureza dos compostos aos quais deve ser atribuída esta atividade.

Nossos testes *in vitro*, com células precursoras hematopoiéticas de sangue de cordão umbilical, comprovaram o efeito da *Uncaria tomentosa* sobre os precursores mieloides, causando um aumento do tamanho das colônias de granulócitos/monócitos e nas colônias mistas.

Considerando a importância do sistema imune na evolução do câncer cólon retal; a influencia do estresse oxidativo na progressão da doença e resistência à quimioterapia; os efeitos colaterais decorrentes do tratamento quimioterápico usando o esquema FOLFOX4 e as propriedades da *Uncaria tomentosa* de estimular o sistema imune (WURM et al., 1998), e, aumentar o número de leucócitos (SHENG et al., 2000a); de apresentar atividade antioxidante (PILARSKI et al., 2006); de reparo ao DNA (MAMMONE et al., 2006; SHENG et al., 2001) de estimular os precursores mieloides (EBERLIN et al., 2005) e não apresentar toxicidade (KEPLINGER et al., 1999; NCCAM/NIH, 2011; SANTA MARIA et al., 1997) foi avaliado seu uso como adjuvante no tratamento do câncer cólon retal. Apesar os dados pré-clínicos demonstrarem resultados animadores, na dose de 300 mg/dia de extrato hidroalcoólico seco, a *Uncaria tomentosa* não foi efetiva nos parâmetros analisados: (i) hemograma, (ii) contagem de linfócitos $CD4^+$, $CD8^+$ e dosagem sérica de IL6; (iii) análise dos níveis de TBARS, proteína carbonil, atividade das enzimas catalase e SOD; (iv) análise dos danos ao DNA pelo ensaio Cometa; (v) redução dos efeitos colaterais da quimioterapia.

A não efetividade do tratamento com *Uncaria tomentosa* em promover reparo do DNA pode estar associada ao tipo de extrato utilizado. Os autores que tiveram resultados positivos nesses parâmetros utilizaram extratos aquosos de *Uncaria tomentosa*, atribuindo o efeito ao ácido quínico (AKESSON et al., 2005; SHENG et al., 2000a; SHENG et al., 2001).

Em relação à atividade antioxidante, apenas um discreto aumento da atividade da SOD foi observado no grupo tratado com *Uncaria tomentosa*, em relação ao início do tratamento, porém não suficiente para representar uma diferença significativa entre os grupos (recebendo ou não *Uncaria tomentosa*). Nossos dados estão de acordo com Pilarski et al. (2006), que demonstraram que o extrato alcoólico (etanol 50%) apresentava importante atividade antioxidante *in vitro*, inclusive com aumento da atividade da SOD.

A falta de resposta na recuperação da neutropenia deve ser observada com relação à intensidade do evento adverso. No modelo animal, onde foi utilizado a ifosfamida como agente causador de neutropenia, ocorreu severa redução no número de neutrófilos e a recuperação foi possível utilizando a dose de 5 mg/dia, que corresponde a uma dose de 1 g/dia para uma pessoa de 60 kg. Já em outro estudo realizado por nosso grupo de pesquisa, com pacientes com câncer de mama, em tratamento com adriamicina e ciclofosfamida, foi observada uma neutropenia discreta, e o grupo que recebeu *Uncaria tomentosa* na dose de 300 mg/dia teve um aumento significativo no número de neutrófilos (dados não publicados). Os pacientes do presente estudo, demonstraram neutropenia de moderada à severa e a dose de 300 mg/dia não foi efetiva.

CONCLUSÕES

__As mulheres apresentaram menores valores de linfócitos T $CD8^+$, menores níveis de Hb e apresentaram risco 2,27 vezes maior que os homens para progressão da doença, com o surgimento de metástases ou aumento dos implantes prévios. Mais estudos são necessários para avaliar uma possível ligação entre essas observações. Esses dados indicam que as variáveis contagem de linfócitos T $CD8^+$ e Hb possam ser consideradas, em conjunto com os demais fatores de risco, para a escolha do melhor esquema terapêutico. O conteúdo de TBARS possui correlação positiva com as enzimas envolvidas no metabolismo glicolítico e da glutaminólise, sendo um marcador precoce da progressão do câncer. Esses dados corroboram a importância do metabolismo glicolítico na formação e progressão das metátases do câncer cólon retal, o que possibilitaria seu uso na avaliação precoce da resposta terapêutica do tratamento quimioterápico adjuvante.

__O extrato seco de *Uncaria tomentosa* foi eficiente na recuperação da neutropenia induzida por quimioterapia em modelo animal, desde a menor dose testada, 5 mg/dia por animal, que equivale a 1 g/dia para uma pessoa de 60 kg. O extrato seco de *Uncaria tomentosa* apresentou uma potência equivalente à 85,2% do medicamento padrão filgrastim (rhG-CSF), nas doses testadas (5 - 15 mg de *Uncaria tomentosa* e 3 – 9 μg de filgrastim), o que permite estabelecer uma dose eficaz para o extrato de *Uncaria tomentosa*. Podemos concluir que a recuperação dos neutrófilos é devido à capacidade da *Uncaria tomentosa* estimular a proliferação dos precursores mieloide, como demonstrado pelo ensaio *in vitro*, com células tronco hematopoéticas de sangue de cordão umbilical.

__Subsequente, o ensaio clínico com pacientes com câncer cólon retal demonstrou que o extrato seco de *Uncaria tomentosa*, na dose de 300 mg/dia, não é efetivo em minimizar a neutropenia causada pelos quimioterápicos 5-FU e oxaliplatina (esquema FOLFOX4). De forma semelhante, o tratamento com *Uncaria tomentosa* não alterou o perfil imunológico (avaliado pela contagem de linfócitos T $CD4^+$, $CD8^+$ e nível sérico de IL6) nem o nível de estresse oxidativo e defesas antioxidantes (avaliado pelos níveis de TBARS, proteína carbonil, enzimas catalase e SOD) e os danos ao DNA.

Portanto, o uso da *Uncaria tomentosa*, como fitoterápico, é promissor para recuperar a neutropenia causada pela quimioterapia, porém mais estudos são necessários para estabelecer

as doses recomendadas, de acordo com a severidade da toxicidade medular do quimioterápico em questão.

PERSPECTIVAS FUTURAS

Estudos subsequentes serão necessários para responder algumas questões: (i) A *Uncaria tomentosa* pode interferir com a atividade dos quimioterápicos, se usada simultaneamente, devido à sua atividade antioxidante? (ii) Por que ocorreu redução do conteúdo de hemoglobina nas culturas que continham 200 mcg de extrato de *Uncaria tomentosa*/mL de meio de cultivo? (iii) Quais vias são ativadas nas células tumorais e nas não transformadas, de forma que a *Uncaria tomentosa* apresente propriedades aparentemente antagônicas: apoptose x proliferação? (iv) Essas propriedades antagônicas são apenas uma questão de dose?

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