Universidade Estadual de Londrina

TATIANE RENATA FAGUNDES

AVALIAÇÃO DO EFEITO POTENCIALIZADOR DA CAFEÍNA SOBRE O ANTINEOPLÁSICO DACARBAZINA EM MODELOS EXPERIMENTAIS DE MELANOMA MURINO

Londrina
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Londrina, 16 de abril de 2015.

Ao meu pai (in memorian)
A Deus, pela oportunidade.

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não é o que nos pagam por ele, mas
aquilo em que ele nos transforma.”
John Ruskin

FAGUNDES, Tatiane Renata. **Avaliação do efeito potencializador da cafeína sobre o antineoplásico dacarbazina em modelos experimentais de melanoma murino.** 2015. 63 folhas. Dissertação (Mestrado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2015.

RESUMO

A quimioterapia potencializada com cafeína vem sendo utilizada em alguns tumores sólidos como osteossarcoma, porém não há relatos de seus efeitos na quimioterapia do melanoma. Esse trabalho investigou o efeito potencializador da cafeína (CAF) em relação ao antineoplásico dacarbazina (DTIC), um agente genotóxico empregado no tratamento do melanoma, em modelos experimentais *in vitro* e *in vivo*. *In vitro*, o protocolo de pré-tratamento (cafeína por 24 horas seguida de dacarbazina por 4 horas) demonstrou maior efeito potencializador, através de testes de viabilidade celular, lesão de DNA e apoptose. O pré-tratamento com CAF acelerou o metabolismo do DTIC e facilitou a geração de estresse oxidativo. A CAF ainda foi capaz de diminuir a marcação da proteína KI-67, reduzir a marcação nuclear de Erk1/2 e aumentar a marcação de p53. *In vivo*, o efeito potencializador da CAF também esteve presente com uma redução de 71,3% dos nódulos tumorais em modelo de metástase pulmonar em camundongos C57/BL6. Concluímos que a cafeína apresenta um efeito promissor nos casos de melanoma, podendo agir como agente coadjuvante na quimioterapia com DTIC

Palavras-chave: Cafeína, melanoma, dacarbazina, tratamento, potencialização.

FAGUNDES, Tatiane Renata. **Evaluation potentiating effect of caffeine on antineoplastic dacarbazine in experimental models of murine melanoma.** 2015. 63 leaves. Dissertation (Master in Experimental Pathology) - State University of Londrina, Londrina, 2015.

ABSTRACT

Chemotherapy potentiated with caffeine has been used in some solid tumors such as osteosarcoma, but there are no reports of its effects on melanoma chemotherapy. This study investigated the impact caffeine (CAF) treatment on the antitumoral effect of dacarbazine (DTIC), an antineoplastic agent used in the treatment of melanoma, in experimental models using murine B16F10 cell line, *in vitro* and *in vivo*. *In vitro*, pre-treatment protocol (caffeine for 24 hours followed by dacarbazine for 4 hours) demonstrated a greater potentiating effect revealed by cell viability, DNA damage and apoptosis tests. Pretreatment with CAF accelerated the metabolism of DTIC and increased oxidative stress generation. CAF was able to reduce Ki-67 labelling and reduce nuclear labelling of ERK1 / 2, and increase p53 nuclear labelling. *In vivo*, CAF potentiation effect was also present with a reduction of 71.3% of tumor nodules in lung metastasis model in C57 / BL6 mice. We conclude that caffeine has a promising effect in melanoma treatment with DTIC.

Keywords: Caffeine, dacarbazine, melanoma, treatment, potentiation.

LISTA DE FIGURAS

LISTA DE ABREVIAÇÕES E SIGLAS

AKT- Proteína quinase B

CDKN2A- Gene supressor de tumor

CDK- Quinase dependente de ciclina

DMEM- Dulbecco's Modified Eagle Medium

DNA- Ácido desoxirribonucleico

DTIC- Dacarbazina

ERK- Quinase regulada por sinal extracelular

FLIP- Proteína inibitória homóloga a FLICE

GSH- Glutationa reduzida

IAP- Inibidor de apoptose

INCA- Instituto Nacional do Câncer

JB6 CL41- Células epidérmicas de rato

LDH- Lactato desidrogense

MAPK- Proteínas quinase ativadas por mitógenos

MAP- Proteínas associadas a microtúbulos

MDA- Malondialdeído

MDC- Monodalzilcadaverina

PCNA- Monodanzilcadaverina

PI3K- Fosfatidilinositol 3-quinase

PTEN- Homológo da fosfatase e tensina

UV-Ultravioleta

SUMÁRIO

1. Introdução

1.1. Melanoma

No Brasil o câncer de pele corresponde a 25% de todos os tumores malignos registrados no País. Dentre os tumores que acometem a pele, o melanoma cutâneo apresenta relativa baixa incidência – correspondendo a 4% dos casos – mas é considerado um dos mais importantes tipos de câncer de pele devido a sua letalidade. Para o Brasil são previstos 5890 novos casos para 2015 (MEIER *et al.*, 1998; BALDI *et al.*, 2003; LAHN *et al.*, 2004; LEVERKUS *et al.*, 2006; INCA, 2015). É uma neoplasia maligna com origem nos melanócitos, células originadas da crista neural e localizadas na camada basal da pele. Em reposta à radiação ultravioleta (UV), os queratinócitos da epiderme secretam fatores que regulam a sobrevivência, proliferação e motilidade dos melanócitos, estimulando a síntese de melanina (GRAY-SCHOPFER; WELLBROCK; MARAIS, 2007). A melanina atua como uma barreira, diminuindo a absorção da radiação UV, além de definir a cor da pele, olhos e cabelos (TADOKORO et al., 2005). Mutações em genes críticos que regulam o crescimento celular, a produção autócrina de fatores de crescimento e a perda de receptores de adesão contribuem na interrupção da sinalização intracelular dos melanócitos, permitindo-lhes escapar de sua regulação controlada pelos queratinócitos (HAASS; SMALLEY; HERLYN, 2004).

1.1.1 – Melanoma: Doença multifatorial

O melanoma é um bom exemplo da origem multifatorial do câncer, uma vez que fatores genéticos e ambientais estão envolvidos. O exato mecanismo molecular que leva a desregulação da homeostasia dos melanócitos é ainda desconhecido. Estudos sugerem que a exposição aguda à radiação UV na infância ou adolescência possa ser responsável por cerca de 40% a 50% de risco de desenvolvimento de melanoma (MILLER e MIHM, 2006). A excessiva exposição à radiação UV tem como consequência a formação de espécies reativas de oxigênio, levando a alteração no equilíbrio redox podendo induzir reações inflamatórias na pele. Já o DNA pode absorver diretamente a luz UVB resultando na formação de fotodímeros de pirimidina (6-4), conhecidos como pirimidona e dímeros de pirimidina (HECK *et al*, 2004).

Dentre os fatores de risco, pode-se considerar o fenótipo da pele, sendo a pele clara a mais susceptível, a presença de nevos melanocíticos, proliferações benignas dos melanócitos, associadas ao surgimento do melanoma malignos, e fatores genéticos familiares (PAEK *et al*., 2008; GARCIA, McLAREN e MEYSKENS, 2011, BAXTER e PAVAN, 2013).

As mutações no melanoma estão relacionadas com casos familiares, e ocorrem principalmente em proteínas reguladoras do ciclo celular (MILLER e MIHM, 2006; PAEK *et al*., 2008; LAZAR e MURPHY, 2010).

Outras mutações envolvidas no surgimento do melanoma são as que ocorrem nos genes N-RAS e BRAF, cujas proteínas estão envolvidas na via de sinalização MAPK (do inglês *Mitogen-Activated proteins kinases*) e PI3K/AKT (do inglês *Phosphatidylinositol-3 kinase/ Protein Kinase B*), sendo que ambas as vias estão envolvidas em sinais de proliferação, adaptação e sobrevivência celular. Mutações em N-RAS estão presentes em 10 a 15% dos casos de melanoma, enquanto as mutações em BRAF aparecem em 60 a 70% dos casos (LAZAR e MURPHY, 2010).

1.1.2 – Melanoma: Aspectos clínicos

Quando diagnosticado precocemente, o melanoma frequentemente apresenta uma lesão inicial na pele sem evidência de metástase. Nesta fase o tratamento é remoção cirúrgica. Porém, quando a doença encontra-se disseminada para linfonodos distantes ou metastatizada, é comum ser refratária às terapias disponíveis, e na maioria das vezes é incurável (EMMETT, DEWING e PRITCHARD-JONES, 2007; KORN *et al.*, 2008).

O melanoma pode ser classificado em quatro padrões de crescimento, de acordo com a localização anatômica do tumor primário e as características histopatológicas da lesão. São eles: Melanoma extensivo superficial, subtipo mais comum, com uma frequência entre 60% e 70% de todos os tipos de melanomas cutâneos (FERNANDES *et al*., 2005), ocorrendo principalmente entre a quarta e quinta décadas de vida e habitualmente surge no tronco em homens e nos membros inferiores em mulheres, estando associado a locais com foto exposição intermitente. Tem início sobre uma neoplasia benigna pré-existente chamada nevo, e tende a apresentar um crescimento radial, com invasão da derme e metástases mais tardias (GOSH e CHIN, 2009). O melanoma nodular representa o segundo tipo mais comum, correspondendo a 15% dos casos. Surge principalmente no tronco e nos membros sobre a pele íntegra, não possui fase de crescimento radial prévio e é caracterizado por uma rápida evolução, com crescimento estimado de 0,49 mm de profundidade por mês. Acomete, normalmente, homens com idade superior a 50 anos (CHAMBERLAIN, 2009). O melanoma lentigo maligno mais comum em idosos, surgindo em área de foto exposição crônica, apresenta-se como uma mancha acastanhada ou enegrecida, de limites nítidos e irregulares, alcançando vários centímetros de diâmetro, frequentemente localizada na face (90%), em mãos e membros inferiores (10%) (CHAMBERLAIN, 2009). O melanoma lentiginoso acral é o subtipo mais raro, sendo mais comum em indivíduos asiáticos, hispânicos e africanos, sem

predileção por sexo. Localiza-se essencialmente nas extremidades (palmas, plantas do pé e leito ungueal,). É o tipo histológico mais agressivo dentre os melanomas (LEDERMAN e SOBER, 1985).

O melanoma pode apresentar dois padrões de crescimento, o radial- crescimento horizontal, ou vertical- crescimento invasivo em profundidade. Os melanomas com padrão de crescimento radial apresentam melhor prognóstico comparado ao padrão vertical. O tipo extensivo superficial apresenta crescimento radial na maioria dos casos, enquanto os tipos nodular, acral-lentiginoso e lentigo-maligno frequentemente apresentam crescimento vertical (THÖRN *et al*., 1994). As características histopatológicas dos dois padrões de crescimento e do aspecto macroscópico do melanoma cutâneo estão ilustradas na Figura 1.

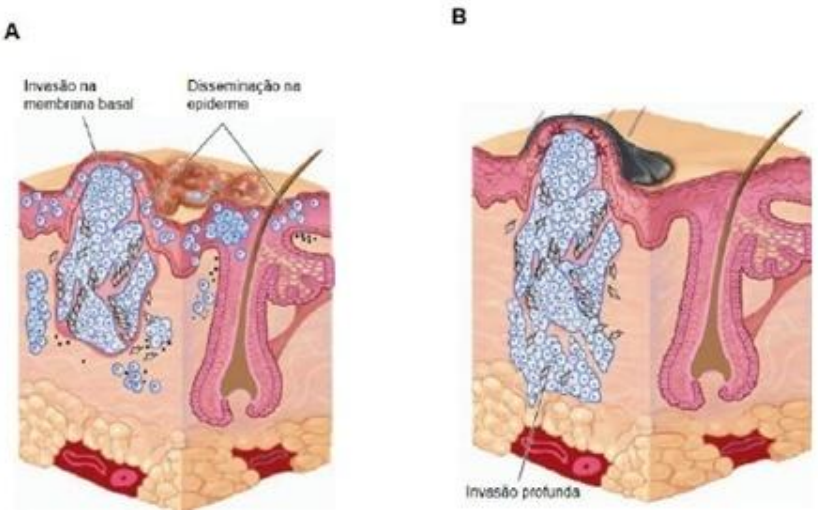

Figura 1. Padrões de crescimento do melanoma. A) Crescimento radial, com invasão de células tumorais na epiderme; B) Crescimento vertical, com invasão profunda da derme. Adaptação: CHIN *et al*., 1998.

A classificação clínico-histopatológica do melanoma cutâneo pode ser realizada através dos níveis de Clark e/ou medida de espessura de Breslow, sendo que atualmente a última tem sido mais utilizada, por apresentar maior correlação com o prognóstico do paciente (SOONG *et al*., 1998; BICHAKJIAN *et al*., 2011). A classificação de acordo com Clark mede o grau de invasão, de I a V, da epiderme (Nível I, tumor in *situ*) à hipoderme (Nível V). A medida de espessura de Breslow mede a profundidade em milímetros. O melanoma é classificado em 5 estágios: Estágio 0 ou melanoma *in situ*; Estágio I, onde a espessura de Breslow do tumor não ultrapassa 2 mm; Estágio II, caracterizado principalmente pela

presença de ulceração em tumores com espessura de Breslow maior que 1 mm; Estágio III, onde o tumor encontra-se disseminado nos linfonodos ou na pele e tecido subcutâneo, com até 2 cm de distância do tumor primário, também chamado de melanoma regional; e Estágio IV, quando a doença encontra-se disseminada para órgãos distantes, também chamada de melanoma altamente disseminado (BALCH *et al*., 2009).

As células metastáticas do melanoma são geneticamente heterogêneas, e os pacientes com doença disseminada apresentam menos de 10% de sobrevida em um período de 5 anos (FRANCKEN *et. al.,* 2008; JACOBS, LANGE, BALCH, 2008). A cirurgia, a quimioterapia e a imunoterapia são oferecidas a pacientes com metástase e são essencialmente paliativas, com baixa previsão de benefício para o paciente (BALCH *et. al*., 2001; DAS TAKHUR *et al*., 2013). Na doença regional, o número de linfonodos acometidos é o fator prognóstico mais importante. Para os pacientes com doença disseminada para órgãos distantes, as únicas variáveis de significado prognóstico são o número de metástases locais, ressecção cirúrgica, duração da remissão e localização das metástases. Apesar dos avanços na quimioterapia, inclusive na imunoterapia, o sucesso no tratamento medicamentoso da doença disseminada permanece com limitações, e o prognóstico da doença metastática é reservado (ASCIERTO et *al.*, 2013; DAS TAKHUR *et al.,* 2013).

O melanoma também pode progredir rapidamente de uma forma branda para uma doença metastática agressiva com alta mortalidade e pobre resposta às terapias, não sendo os mecanismos de progressão e resistência aos agentes terapêuticos bem elucidados (JEFFS *et al.*, 2009).

1.1.3 – Melanoma: Tratamento

Atualmente, o regime padrão de tratamento para o melanoma inclui a retirada do tumor por cirurgia, seguida de quimioterapia, de imunoterapia e/ou de radioterapia. Entretanto, nem todos respondem à terapia, e muitos pacientes têm recaída ou progressão da doença (SOMASUNDAR *et al*., 2005). Apesar de ser uma doença com grande capacidade para produzir metástase, o melanoma pode apresentar cura quando detectado no estágio precoce. A ocorrência de metástases normalmente leva à sobrevida média de apenas seis a nove meses, tornando-o o terceiro tumor metastático mais comum, depois dos cânceres de pulmão e de mama (EWEND *et al*., 1996; JOHNSON e YOUNG, 1996; TARHINI e AGARWALA, 2004).

Células de melanoma apresentam baixos índices de apoptose espontânea *in vivo* em comparação com outros tipos de células tumorais, e são relativamente resistentes à apoptose induzida por agentes quimioterápicos. Dois membros das famílias de proteínas inibidoras da

apoptose IAP (Do inglês *Inhibitor of Apoptosis*) e FLIP (Do inglês *FLICE-like Inhibitory Protein*) têm sido associados com a progressão do melanoma, sendo detectáveis no início da disfunção melanocítica (nevos melanocíticos) e em maior quantidade em melanomas metastáticos altamente invasivos (SOENGAS e LOWE, 2003).

O gene da proteína p53, considerado como o "guardião do genoma", é ativado em resposta a sinais de dano celular, promovendo a parada do ciclo celular na fase G1, portanto, antes de ocorrer a duplicação do DNA (fase S), permitindo o reparo do DNA danificado (FEET-CONTE e SALLES, 2002). Mutações nesse gene têm sido relacionadas com esta resistência a apoptose, sua frequência em melanomas é bastante divergente, mas estas mutações estão relacionadas com a transformação maligna dos nevos melanocíticos (SOENGAS e LOWE, 2003; HOUBEN et al., 2011). Avery-Kiejda e colaboradores (2011) demonstraram atividades reduzidas da p53, com perda da capacidade de regular os seus genes alvo, envolvidos no controle do ciclo celular e apoptose, em amostras de melanoma metastático e em linhagens celulares de melanoma cultivadas *in vitro*, especialmente em casos de resistência a quimioterapia.

Atualmente ainda não há um agente terapêutico conhecido que proporcione o prolongamento da vida dos pacientes com melanoma metastático. As estratégias quimioterápicas têm sido baseadas no uso de dacarbazina, na forma de monoterapia, que é o fármaco mais ativo para a doença, com resposta terapêutica em torno de 10% a 20% (TARHINI e AGARWALA, 2006). Já em casos mais extremos de metástase se utiliza a temozolomida (MIDDLENTON *et al*., 2000).

A dacarbazina é um pró-fármaco que exige ativação inicial pelo citocromo P450, através de uma reação de N-desmetilação, o composto resultante é subseqüentemente clivado na célula-alvo, liberando um derivado alquilante que lesa o DNA não permitindo a replicação correta e levando a quebra de cadeias de DNA e morte celular (SLAPAK e KUFE, 1994; COLVIN, 2001; COLOMBO *et al.*, 2001). Os efeitos adversos consistem em mielotoxicidade, náuseas e vômitos intensos (RANG *et al*., 2003).

Na tentativa de melhorar a resposta terapêutica alguns protocolos sugerem a combinação da dacarbazina com outros quimioterápicos, no entanto esta associação tem efeitos contraditórios e alguns autores apontam um aumento da toxicidade sistêmica sem uma potencialização efetiva do tratamento (SASSE *et al*., 2007; LUI *et al*., 2007).

Apesar de a dacarbazina ser o tratamento de primeira linha para melanoma metastático, novas terapias tem sido avaliadas, como no caso do uso de anticorpos

monoclonais, como o ipilimumab (anti-CTLA4) e vemurafenibe (anti-BRAF), recentemente aprovados para o tratamento (ROBERT *et al*., 2011).

1.2. Cafeína

A cafeína é o mais conhecido constituinte do café devido às suas propriedades fisiológicas e farmacológicas. É um alcalóide farmacologicamente ativo pertencente ao grupo das xantinas, altamente resistente ao calor, inodoro e com sabor amargo bastante característico que contribui de forma importante para o sabor e aroma do café (MONTEIRO e TRUGO, 2005). Seu consumo de baixas a moderadas doses pode resultar em efeito estimulante do sistema nervoso central, com possível diminuição do sono e aumento na capacidade de concentração. No entanto, em altas doses e em indivíduos com sensibilidade aumentada, a cafeína pode causar efeitos negativos como ansiedade, inquietação, insônia e taquicardia (NEHLIG, 1999). Essas propriedades estão relacionadas com sua forte semelhança estrutural com a adenosina, competindo com seus receptores e impedindo assim sua ação como redutora da frequência cardíaca, da pressão sanguínea e da temperatura corporal, fatores responsáveis pela sensação de torpor e sono (NAWROT *et al*., 2003). Além de exercer efeito sobre o sistema nervoso central, a cafeína é outro constituinte do café com atividade antioxidante, que a torna capaz de inibir a degradação oxidativa, e também é efetiva inibindo a peroxidação lipídica, um processo definido como uma cascata de eventos bioquímicos resultantes da ação dos radicais livres sobre os lipídeos insaturados das membranas celulares, levando à destruição de sua estrutura, falência dos mecanismos de troca de metabólitos e, numa condição extrema, à morte celular (BENZIE, 1996). Esse efeito da cafeína foi observado *in vitro,* induzido por espécies reativas de oxigênio em microssomos de fígado de rato, sendo seu potencial antioxidante semelhante ao da glutationa e superior ao do ácido ascórbico (DEVASAGAYAM *et al*., 1996).

A cafeína é uma xantina (1,3,7- trimetilxantina), naturalmente presente em várias plantas distribuídas por todo o mundo. No entanto, a principal fonte de cafeína é o café (*Coffe arabica* L.), com mais de 60 espécies conhecidas pelo mundo (SUZUKI e WALLER, 1988). As fontes naturais mais comuns de cafeína são: o café, a semente de cacau (utilizada na fabricação do chocolate), a semente de cola (utilizada na fabricação de refrigerantes) e os chás. A quantidade de cafeína nos alimentos varia dependendo do tamanho da porção, do tipo do produto e do método de preparo (NAWROT *et al*., 2003).

Já foi relatado que tratamento de células em cultura com cafeína afeta o ciclo celular e sua regulação de várias formas, afetando proteínas regulatórias, induzindo apoptose de forma

dependente e independente da proteína p53 (BODE e DONG, 2007). A cafeína afetou seletivamente as células de carcinomas do tipo escamoso ou espinocelular sem afetar células vizinhas normais na pele (LU *et al*., 2002); o tratamento com cafeína por 24 horas em células epidérmicas de rato, JB6 CL41, afetou o ciclo celular induzindo fosforilação de p53 e apoptose associada ao aumento da expressão de proteína pró- apoptótica Bax e caspase 3, uma enzima efetora da via apoptótica (HE *et al*., 2003). Sobre células de carcinoma metastático de pulmão humano a cafeína induziu apoptose, e atuou sinergicamente aumentando a apoptose induzida por radiação, e também induziu parada em G1 do ciclo celular, revelando que a cafeína tem efeitos sobre diferentes quinases dependentes de ciclina (QI, W. *et al*., 2002). Células da linhagem de leucemia promielocítica HL-60, foram expostas a grandes quantidades de irradiação (3,9 mGy / min), que resultou no acúmulo de células na fase G2 do ciclo celular; o tratamento com cafeína eliminou essa parada no ciclo celular em G2, levando as células à apoptose (VAVROVA *et al.*, 2003).

A cafeína também demonstrou atividade contra hepatocarcinogênese *in vitro,* induzida por dietilnitrosamina em ratos; reduzindo a expressão de alguns marcadores celulares de hepatocarcinoma, como o antígeno nuclear de proliferação celular (*proliferating cell nuclear antigen*- PCNA) e a enzima a *glutationa-S-tranferase* (GST) (FUJISE *et al.,* 2011).

Gude e colaboradores (2001) avaliaram o efeito isolado da cafeína sobre melanoma metastático e local induzidos experimentalmente. A administração via oral de cafeína na dose 100mg/kg mostrou uma redução significativa no volume do tumor sólido e no tumor metastático do pulmão.

Em estudos *in vivo,* Kawahara e colaboradores (2008) demonstraram a potencialização da cisplatina em animais com metástase pulmonar de osteossarcoma tratados também com cafeína 3 vezes ao dia com doses variando de 30 a 100 mg/Kg e também observou maior redução das metástases em animais tratados com co-administração de cafeína.

A cafeína tem sido descrita desde os anos 1980 como potencializadora dos efeitos citotóxicos de drogas antineoplásicas, através de seu efeito de inibição de reparo de DNA (TSUCHIYA *et al*., 1994). Já foi demonstrado que a quimioterapia com doxorrubicina, cisplatina e ifosfamida potencializada por cafeína induziu melhora em pacientes com osteossarcoma, com aumento das taxas de resposta de 86% em casos relatados. (TSUCHIYA H. *et al*., 1999). Karita e colaboradores (2011) avaliaram o efeito da quimioterapia com cisplatina e doxorrubicina combinada com cafeína em 5 pacientes com sarcoma de células claras; todos os pacientes sobreviveram, e apenas um obteve recidiva em um acompanhamento médio de 45 meses. O mesmo efeito foi observado por Takauchi e

colaboradores (2007) em 90 pacientes com sarcoma de tecido mole, que obtiveram taxa de sobrevida global de 5 anos em 80,7% dos pacientes, em um período de acompanhamento de 52 meses. Miwa e colaboradores (2010) reportaram em um caso de tumor células redondas desmoplásicas, um tumor raro que ocorre na cavidade abdominal que o tratamento com quimioterapia potencializada por cafeína e sem remoção cirúrgica, dizimou o tumor, sem recidiva e metástases locais em um período de acompanhamento de 4 anos.

2. Justificativa

Vários autores afirmam que a quimioterapia assistida pela cafeína é uma forma segura e mais eficaz de tratamento, com diminuição dos fortes efeitos colaterais que surgem com a administração de drogas antineoplásicas (Tsuchiya *et al*., 1998). Embora Gude e colaboradores (2001) tenham relatado efeito potencializador da cafeína no modelo de melanoma murino metastático, não há trabalhos demonstrando o efeito potencializador sobre a dacarbazina, o quimioterápico mais comumente utilizado no tratamento do melanoma metastático.

Uma vez que a cafeína tem demonstrado efeito adjuvante no tratamento de tumores sólidos, potencializando a ação dos quimioterápicos, deve-se investigar se CAF também é capaz de agir em casos de melanoma, uma vez que este é um tipo de câncer muito resistente às terapias tradicionais, necessitando ainda esclarecer os mecanismos de ação envolvidos.

3. Objetivos

3.1. Objetivo Geral

Investigar o efeito potencializador da cafeína sobre o antineoplásico dacarbazina, um agente genotóxico utilizado no tratamento do melanoma, através de modelos experimentais *in vitro* e *in vivo*.

3.2. Objetivos específicos

- Avaliar o efeito da cafeína diretamente sobre a linhagem celular de melanoma murino B16F10;
- Avaliar o efeito do tratamento com cafeína e dacarbazina associadas no pré e pós-tratamento com dacarbazina;
- Avaliar o efeito potencializador do tratamento através de testes de viabilidade celular e lesão nuclear, sobre a linhagem celular de melanoma murino B16F10;
- Avaliar a interferência da cafeína sobre importantes vias de sinalização celular envolvidas no melanoma;
- Avaliar o efeito do tratamento concomitante da cafeína e dacarbazina, através de modelo experimental de melanoma metastático murino.

4. Conclusões Gerais

Esta dissertação teve como produto a confecção de um artigo cientifico e, de maneira geral, nos permitiu chegar às seguintes conclusões:

A cafeína em concentrações séricas (1-10μM, equivalentes ao consumo de café, 20-40 μM, equivalente à suplementação ou medicamentos com cafeína) foi capaz de induzir citotoxicidade nas células de melanoma B16F10 através do aumento da autofagia e com a indução de apoptose. A diminuição da marcação imunocitoquímica para a proteína KI67 aponta para a diminuição na proliferação celular, e o aumento da marcação para a proteína p53 revela que a cafeína aumenta a apoptose e a autofagia. Além disso, a associação de cafeína e dacarbazina aumentou o efeito citotóxico da droga através da diminuição da viabilidade e contagem celular.

No ensaio *in vivo*, o tratamento com cafeína e dacarbazina diminuiu o número de nódulos metastáticos pulmonares em comparação com o grupo tratado apenas com o quimioterápico, mostrando que a cafeína potencializou o efeito do medicamento.

Concluímos que a cafeína apresenta um efeito potencializador no tratamento do melanoma, podendo agir como agente preventivo ou terapêutico, sendo necessários novos estudos para confirmar seus efeitos biológicos neste tipo de tumor.

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Anexos

Anexo A

Artigo: CAFFEINE POTENTIATES THE ANTITUMOR EFFECT OF DACARBAZINE IN MURINE MELANOMA MODELS.

CAFFEINE POTENTIATES THE ANTITUMOR EFFECT OF DACARBAZINE IN EXPERIMENTAL MURINE MELANOMA

Abstract
Caffeine-assisted chemotherapy has been used in some solid tumors such as osteosarcoma, but there are few reports of its effects on melanoma chemotherapy. This study investigated the impact caffeine (CAF) treatment on the antitumoral effect of dacarbazine (DTIC), an antineoplastic agent used in the treatment of melanoma, in experimental models using murine B16F10 cell line, *in vitro* and *in vivo*. *In vitro*, pre-treatment protocol (caffeine for 24 hours then dacarbazine for 4 hours) demonstrated a greater potentiating effect revealed by cell viability, DNA damage and apoptosis tests. Pretreatment with CAF accelerated the metabolism of DTIC and increased oxidative stress generation. CAF was able to reduce Ki-67 labelling and reduce nuclear labelling of ERK1 / 2, and increase p53 nuclear labelling. *In vivo*, CAF potentiation effect was also present with a reduction of 71.3% of tumor nodules in lung metastasis model in C57 / BL6 mice. We conclude that caffeine has a promising effect as a pre-adminstred drug in melanoma treatment with DTIC.
Keywords: Caffeine, melanoma, dacarbazine, potentiation, treatment

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1. Introduction

Caffeine is a xanthine found in coffee that is well-known by its stimulating action on the central nervous system. In the past years, caffeine was emerged by its therapeutic potential, which includes its antitumoral effects. Some of the proposed anticancer mechanisms include the inhibition of cell cycle regulatory proteins and induction apoptosis.[1]

Studies have highlighted caffeine by its capacity to potentiate chemotherapy, as observed in patients with osteosarcoma treated with cisplatin, ifosfamide and doxorubicin[2]. Promissing results have been reported. The association of chemotherapy plus caffeine increased 80.7% of survival in patients (up to 5 years) with non-metastatic sarcoma, withouth increasing adverse effects(such as bone marrow depression) shown in adjuvant treatment with classic anticancer agents[3]. Karita *et al.*[4] evaluated the effect of chemotherapy with cisplatin and doxorubicin combined with caffeine in 5 patients with clear cell sarcoma; all patients survived, and only one did recurrence at a median follow-up of 45 months. Miwa *et al.*[5] reported a case of desmoplastic round cell tumor treated with caffeine-assisted chemotherapy, the tumour was removed, without recurrence and local metastasis in a follow-up period of 4 years.

Some authors consider the association of caffeine with antineoplastic chemotherapy a more effective and safest form of treatment, that can potentially be used to treat different types of cancer[6,7]. One important mechanism of potentiation is related to DNA repair inibition by caffeine treatment[8]. Gude *et al.*[9] evaluated the isolated effect of caffeine on metastatic melanoma and local induced experimentally in mice. The oral administration of caffeine in a dose 100 mg / kg showed a significant reduction in tumor volume of the solid and metastatic lung tumor. Therefore, the use of caffeine associated with chemotherapeutic drugs seems a promising strategy, especially in cases of scarse therapeutic options and poor clinical response.

In this context, one of the cancers with lower therapeutic response is cutaneous melanoma, the most aggressive solid tumors prone to metastasis. Melanoma has a low global incidence, for 2015 are predicted 137,310 new cases of melanoma[11], but the mortality is high, especially when it is not early diagnosed[11]. The treatment of melanoma includes surgery removal of tumor, followed by monotherapy with dacarbazine, with low therapeutic response rates (10% to 20% of patients)[12], and many patients have a recurrence or progression of the disease[13].Another related problem is metastasis, that usually leads to median survival (six to nine months), making it the third most common metastatic tumor, after lung and breast cancers[14,15]. Considering this, the present study aimed to evaluate the effects of the association of caffeine with the antineoplasic drug dacarbazine by using *in vitro* and *in vivo* approaches.

2. Materials and Methodos

2.1. *In vitro*

Cell culture and treatments

The murine melanoma cell line B16F10 was cultivated in culture flasks with Dulbeco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% mixture of penicillin / streptomycin (St. Cruz). The culture in the incubator (Sanyo, Japan) humidified at 37 ° C with 5% CO_2. Tests for cytotoxicity, and genotoxicity proliferation were performed in 24 well plates with $2x10^5$ cells / well and maintained in the incubator for 24 hours for adhesion and cell stabilization.

We used two protocols to evaluate potentialization effect, pre- and post-treatment. For pre-treatment, the cells were treated with different doses of caffeine (CAF 5, 10. 20 and 40 µM-24 hours), according to Zheng e Williams (2002)[16] before exposure to dacarbazine (DTIC250 and 500 µM- 4 hours) (Jin et al., 2011)[17]. For the post-treatment, cells were exposed to dacarbazine (DTIC250 and 500 µM) for 4 hours and then were treated with

caffeine (CAF 5, 10, 20 and 40 µM) for 24 hours. The cells were washed with PBS before receiving the next treatment. We also evaluated effects of caffeine (24h) on B16F10 cells. All experiments were performed in triplicate and three independent replicates.

Determination of IC_{50} of the drug concentration in B16F10 murine melanoma cells

The determination of the IC_{50} inhibitory concentration was obtained from the logarithmic regression of the MTT ((3- (4,5-mymethylthiazol-2-yl) -2,5-dyphenyltetrazolium bromide) test results. MTT assay was performed according to Mosmann[18], and the results were also used to describe cell viability.

Cell proliferation and cell viability

After the treatment, the cells were washed with phosphate-saline solution (PBS) and trypsinized, suspended in Trypan blue (0,05%) and counted using a Neubauer chamber. Cell counts in all four external quadrants of the chamber, allows identification increased or decreased cell proliferation. For cell viability were counted a total of 300 cells, distinguishing viable cells (stained in blue).

Comet assay

Comet assay allows to evaluate damage to the genetic material, and was performed according to the protocol described by Singh *et al.*[19]as modified by *Tice et al.*[20]. After the treatments, cells were tripsinized and suspended in low melting point agarose. The cells were transfered to agarose slide. The cells were submitted to lysis solution before electrophoresis with alkaline pH. After fixation of the cells, slides were analyzed at Olympus Fluorescence Microscope System BX3-URA (Olympus Corporation, Tokyo, Japan), using Gel Red staining. The obtained pictures (100x) of the nucleoids were analysed in Comet Score™

Freeware program (Tritek, USA) to determine tail moment score. The tail moment was regarded as DNA damage index. The slides were photographed using Olympus Fluorescence Microscope System BX3-URA (Olympus Corporation, Tokyo, Japan) at 100x magnification, photographed of 20 random fields were analyzed, all cells of a field were counted and the percentage of cells labeling with light core brown was estimated.

The comet assay was used for synergism analysis, using the tail moment as a response to tested caffeine and dacarbazine concentrations. We used statistic factorial design of 2^2 with 9 genuine replicates for each point, in order to increase the degree of freedom of the proposed mathematical model. Statistical analyzes were performed using the Statistic software 8.0 Statsoft®.

Lactic dehydrogenase quantification (LDH)

After treatment the culture medium was collected for assaying LDH. The kit for the quantification of LDH (Doles, Goias, Brazil) was used to measure the release of LDH by the damaged cell cytosol into the culture medium, and estimate the membrane damage associated with necrosis. The test is based on the reduction of NAD (nicotinamide adenine dinucleotide) by LDH. Quantification was done following manufacturer is instructions and quantifyed in a microplate reader spectrophotometer (MultiscanGo; Thermo Scientific, USA) at 510 nm.

TUNEL (The DeadEnd™ Colorimetric) assay

After the treatment, the cells grown on circular coverslip were washed and fixed in 10% formaldehyde. Apoptosis was assessed using deadend Colorimetric TUNEL System (Promega, WI, USA) according to manufacturer's protocol. The slides photographed using Olympus Fluorescence Microscope System BX3-URA (Olympus Corporation, Tokyo, Japan)

at 100x magnification, photographed of 20 random fields were analyzed, all cells of a field were counted and the percentage of cells labeling with light core brown was estimated.

Determination of dacarbazine levels in cell culture by HPLC method

To determine the effect of residual dacarbazine a new experiment was performed with $2x10^5$ cells/well undergoing treatment with dacarbazine or pre-treatment (CAF10, 24h) in the same conditions as the other experiments. As a control, DTIC (250 µM) was diluted in culture medium without cells, and maintained under the same conditions until the sample processing. After treatment, cells were collected from the culture medium to analyze dacarbazine levels. The chromatographic analyzes were performed on HPLC (High-Performance Liquid Chromatography) Waters, Alliance e2695 model equipped with a diode-array detector (PDA) and Waters 2998 autosampler. The chromatographic column used consists of a Waters Xterra C18 (5mm, 4.6x250mm). The analytical method was isocratic, the mobile phase consisted of: 12.5% acetonitrile and water acidified with acetic acid to pH 3,05, the running flow was 0.8 ml/min and the injection volume was 10µl. The wavelength used for detection of DTIC was 323 nm.

Determination of malondialdehyde (MDA) levels

MDA is the final product of lipid peroxidation, and was evaluated to determine the involvement of oxidative stress. After the treatment, cells were washed and trypsinized. Cellular suspension were 60xG centrifuged (4°C, 3 minutes). The cellular pellets were suspended in cold PBS buffer and frozen (-80°C) until analyzes. The cellular suspension was used to measure MDA levels. The measurement was performed as described by Victorino *et al* [21] using a HPLC system (Shimadzu20AT-HPLC) equipped with a pump LC20AT, an absorbance detector (diode-array UV SPM20A), and the reverse phase C18 column. Results

were expressed in MDA nM/g of the total protein. The total protein content was determined based on the method described by Lowry and modified by Miller[22].

Autophagy test (Monodanzilcadaverina - MDC)

For this test, the cells were cultured in a circular coverslip. At the end of the treatment, MDC was added to culture medium, according to the protocol described by Biederbick*et al*[23]. The photomicrographs were obtained by the same procedure described for TUNEL test, and were used to the calculated percentage of cells containing cytoplasmatic autophagic vacuoles.

Immunocytochemistry

To identify the effect of caffeine on cell signaling pathways, the use immunocytochemistry to p53, ERK1/2, AKT and KI67. To perform the technique were prepared slides containing representative adherent cells from each treatment with caffeine, fixed in commercial mixture of polyethylene glycols- Citofix® (Doles, Goias, Brazil). Immunocytochemistry analysis was performed using the streptavidin-biotin method LSAB kit (Dako, Japan). Slides were incubated with Triton X-100 solution 10% by washing with PBS and treated for 40 minutes with fetal bovine serum (Santa Cruz), and incubated overnight at 4°C with primary antibodies (anti-p53 (Antibody FL-393: sc-6243, Santa Cruz Biotechnology, Inc.®), anti-ERK1/2 (Antibody Thr 202: sc-101760, Santa Cruz Biotechnology, Inc.®), anti-Ki-67 (Antibody M-19: sc-7846, Santa Cruz Biotechnology, Inc.®) and anti-AKT (Antibody Ser 473-R: sc-7985-R, Santa Cruz Biotechnology, Inc.®), diluted 1: 100. After washing with PBS was applied to each sample biotinylated secondary antibody supplied in the kit (LSAB Dako®) in a humid chamber, followed by application kit streptavidin-peroxidase conjugate and subjected to development with substrate-chromogen (DAB LSAB Kit®, Dako), counterstained with Harris hematoxylin subsequently washed in

water and dehydrated in alcohol graduation (70%, 85% and 100%), cleared in xylene and mounted and covered with cover slip using mounting means of non-aqueous. The slides photographed using Olympus Fluorescence Microscope System BX3-URA (Olympus Corporation, Tokyo, Japan) at 100x magnification, photographed of 20 random fields were analyzed, all cells of a field were counted and the percentage of cells labeling with light core brown was estimated. Mounted slides were then analyzed to obtain quantitative score nuclear immunostaining, or cytoplasmic membrane via Image J software (NIH, USA) using the color deconvolution tool.

Statistical Analysis

The data were expressed as average standard deviation and analyzed by One-way analysis of variance (ANOVA). The differences between groups of cellular counting experiments and viability by Trypan blue, MTT were analyzed with Dunnett`s post-test, for comparisons between different treatments, and the comet assay experiments, LDH autophagy, TUNEL, *in vivo* treatment analysis and MDA by Bonferroni`s test for comparisons between the different doses of the same treatment. For all the significance level was set at $p<0.05$ was statistically Significant Considered. Data analysis was conducted using GraphPad Prism (version 5.0, California, USA).

2.2. *In vivo* study

Experimental animals

The C57BL6 mice (8-12 weeks old, male and female) were housed in standard conditions of temperature and humidity, in collective cages with special food for mice (Nuvilab®) and water *ad libitum*. All procedures were performed after approval by the Ethics

Committee on the use of animals of State University of Londrina, according to the Brazilian Code of recommendations for the use of laboratory animals (No. 14636.2011).

Experimental model

Murine B16F10 melanoma cells were cultured *in vitro* under the same study conditions and inoculated intravenously via ophthalmic plexus at a concentration of $1x10^5$cells in 50µL of DMEM without serum. The control group was inoculated with serum-free sterile medium. The animals were divided into 5 experimental groups; 1- control, without melanoma implantation, 2 - melanoma group, 3- caffeine, animals were served with caffeine diluted in water (0.44 mg/ml- oral administration) *ad libitu* (65 mg/Kg/day), starting at day 4 after B16F10 inoculation, 4 - dacarbazine, animals were treated with DTIC (40mg/kg- intraperitoneal administration) consecutive days starting at 4º day after B16F10 inoculation, 5 - pre-treatment, animals received caffeine before DTIC treatment. Groups that were not treated with DTIC, received saline i.p. injection, and groups that were not treated with caffeine received sterile water.

At day 18 after B16F10 inoculation, the mice were anesthetized with ether and euthanized by cervical dislocation. The lung was removed, washed in potassium phosphate monobasic buffer 10 mM pH 7.4. The number of tumour nodules was immediately determined, each black dot was considered a colony of metastasis, and scored. The animal weight variation (weight at day 18 – weight at day 1) was also evaluated.

Control groups and melanoma in each experimental time were compared by unpaired Student's t test, with $p < 0.05$. For the analysis of weight was used paired t-test, comparing initial and final weight and $p < 0.05$ was considered significant.

3. RESULTS

Firstly we assessed the viability of B16F10 cells using the MTT assay to verify the potentiating effect of CAF on the DTIC and choose the best treatment protocol. In the post-treatment protocol we observed the potentiation effect for the association of DTIC250 with CAF10 and CAF20, but not for DTIC500 treatments (Fig. 1A).

For the pre-treatment protocol, DTIC 250 and 500 were potentiated by all tested concentrations of caffeine (Fig. 1B).Considering this result, we chose the pre-treatment protocol to perform further experiments. Fig. 1B, also shows that potentialization is related to reduction in cell counting and viable cells.

When treated with caffeine, B16F10 reduces cell viability in CAF20 and CAF40 treatments, the IC_{50} 24h was estimated in 39,3μM (Fig. 2A). As the concentration of 40μM is higher than IC_{50} it was not used for other tests. CAF20 reduced the number of viable cells. CAF 10 e CAF 20 reduced the cell counting. As observed, CAF 10 reduced the number of cells without affecting cell viability. As the CAF1 did not showed any biological effect, it was also excluded from other tests.

As the DITC is a known genotoxic agent, we performed the comet assay. For this assay was used for the pre-treatment protocol (Fig. 3A). All tested concentrations of caffeine were not able to generate DNA lesion. DTIC250 was not able to induce DNA damage, but the association with CAF 10 e CAF20 showed genotoxic effect. DTIC 500 was genotoxic, but its genotoxicity was not potentiated by caffeine. The model showed that DNA damage increase is associated with higher concentrations of caffeine, when it is associated to DTIC, p> 0:05.

With respect to the cell death pattern (Fig. 4), it is possible to observe that necrosis occurs only in the association of DTIC 500 with CAF 20 (Fig. 4A). For apoptosis, CAF 20 was able to induce this death pattern in B16F10 cells. Apoptosis was not observed for DTIC 250, but when this concentration of dacarbazine is associated with caffeine we can note

apoptosis in a dose-dependent response. DTIC 500 induced apoptosis, but only the associations with CAF 5 and CAF 10 increased the percentage of apoptotic cells.

In our HPLC method conditions we observed the formation of DTIC peak with a retention time of 3.0±0.2 min (Fig. 5A). It is possible to identify that naïve (without treatment with caffeine) and pre-treated (CAF 10) cells significantly reduced DTIC levels, showing the ability of B16F10 to metabolize DTIC. Naïve cells reduced 18.7% of the DTIC levels, on the other hand pre-treated cells CAF10 reduced 32.9% of DTIC concentration.

Caffeine increased oxidative stress in a dose-dependent manner (Fig. 5B), and increased. Malondialdehyde levels increased for the associations with DTIC 250. CAF 10 and CAF 20 were also able to induce autophagy (Fig. 6A). The caffeine (CAF 5 and CAF 10) did not affect cytoplasmic levels of ERK 1/2and AKT proteins (Fig. 6B and C), but reduced the percentage of nuclear labeling for ERK 1/2 (Fig. 6B). The nuclear p53 labeling was increased by CAF 5 and CAF 10 treatment (Fig. 6D), and CAF 10 decrease KI67 labeling (Fig. 6E).

To evaluate the potentialization of effect of caffeine, we conducted an *in vivo* model of pulmonary metastasis with the implementation of B16F10 cells. All melanoma experimental groups (treated or untreated) lost weight compared to the control group without melanoma (Tab. 1). The smallest losses were observed in the groups treated with caffeine (-2.9 ± 0.7g) and dacarbazine (-2.7 ± 0.7g), and the pre-treatment group (-3.1 ± 0.5g) compared to the control with melanoma (-5.7 ± 0.5g).The experimental group with melanoma developed 128.0 ± 3.0lung tumournodules, the group treated with caffeine showed 90.6 ± 18.2 nodules (29.2% reduction compared to untreated melanoma group); the group treated with DTIC developed 63.6 ± 10.7 nodules, down 53.8%. The highest percentage of inhibition of tumor nodules were observed in the pre-treatment group (71.3% reduction). The differences were completely clear to the naked eye, as observed in Fig. 7A.

4. DISCUSSION

Caffeine has been evaluated as potentiating agent chemotherapy since 1989[24,25,26] with promising results for some tumors, especially osteosarcoma. Several mechanisms have been proposed, including induction of apoptosis and deregulation of proteins involved in the control of cell cycle[27,28]. In spite of this, no previous study has reported the effect of this association in experimental melanoma.

This study evaluated the effect of caffeine on dacarbazine, a drug studied in various melanoma models[29,30]. In our work we used two different protocols of treatment (Fig. 1). The pre-treatment showed the best results, suggesting that caffeine induces cellular changes that are involved in the potentiation effect.

In the pre-treatment protocol we observed potentiation of DTIC by caffeine for decreased cell counting and number of viable cells, suggesting an increase in cytotoxicity (Fig. 1B). On the other hand, the treatment with CAF 10 (Fig. 2) revealed reduced cell counting without reduction of viable cells, also suggesting cytostatic mechanisms, that was confirmed by the decrease in Ki-67 labeling (Fig. 6E), a known marker of cell proliferation.[31]

Okano *et al.*[32]have reported inhibitory effects of caffeine on cell proliferation in several kinds of hepatocarcinoma (HepG2, HLF, Huh7 and PLC / PRF5). The proposed mechanisms involved the inhibition of cell cycle regulation kinases. In this study we evaluated two important cell signaling pathways, through ERK1/2 (involved in the MAPK pathway) and Akt (involved in Akt/ PI3K pathway). In our work, the caffeine-treated cells showed nuclear staining decreased ERK1/2 (Fig. 6B), suggesting that caffeine prevents the translocation of the protein to the cell nucleus. Merighi *et al.*[33] demonstrated that caffeine inhibits the phosphorylation of ERK1/2 in HT29 cells. This report explains our results, once ERK1/2 only translocates to the nucleus after undergoing phosphorylation[34]. Merighi *et al.*[33] have also shown that caffeine inhibits the phosphorylation of AKT, this mechanism can notbe

excluded, since the AKT antibodies used in imunocytochemistryof our work do not distinguish between fosforilated and no fosforilated AKT.

For CAF10 and CAF20 association with DTIC250 increasedgenotoxic effect of dacarbazine (Fig. 3A), that was not observed for DTIC500, suggesting that caffeine really modifies cellular responses to potentiate DTIC. The DTIC (dimethyl triazene imidazole carboxamide) is a synthetic antineoplastic agent purine analogue (5-amino-lH-imidazole-4-carboxamide), which acts through inhibition of DNA synthesis and as an alkylating agent[35], causing DNA lesions. It also needto be activated by biotransformation at microsomal system by CYP1A2[36]. It is possible to conclude that, DTIC requires viable cells to exert genotoxic effect and explains why DTIC 500 (cytotoxic concentration) showed worst potentiation responses.

Caffeine is also metabolized by CYP1A2 enzyme[37]. In rat hepatocytes treated *in vitro* with caffeine, an increase in CYP1A2 expression was observed[38]. Our results indicate that B16F10 is also capable of expressing CYP1A2, and to respond to caffeine treatment, once we observed different percentages of DTIC levels between naïve and pre-treated cells (Fig. 5A). The higher metabolization of DTIC in pre-treated cells, also helps to explain the higher genotoxic effect with the association of DTIC 250 with caffeine, once the metabolite of DTIC is the responsible to DNA damage.

We observed the involvement of oxidative stress in the studied mechanisms of caffeine. The generation of reactive oxygen species is a possible mechanism that may explain the observed increase in MDA levels (Fig. 5B) in B16F10cells.

Augmented MDA suggests that the chain of lipid peroxidation was started in some instance. Although the antioxidant property of caffeine is well-known, Shukla and Gude demonstrated that this substance is able to exert pro-oxidant effects on melanomacells. In fact, caffeine treatment enhances lipid peroxidation and depletes glutathione storage, which has

been associated with its antineoplasic effect in melanoma cells lines. Therefore, the enhanced lipid peroxidation observed here may contribute to cell death, since oxidative stress-derived metabolites are frequently enrolled in genotoxicity, cytotoxicity and apoptosis.

In our study, CAF 20 induced apoptosis (Fig. 4B). For associations we observed increasedpercentage of apoptotic cells, except for the association DTIC500 with CAF20 (the unique association with necrotic pattern of cell death). In response to DNA damage, cells activate enzymes and ATR (ataxia telangiectasia related), which initiate a cell cycle arrest by p53 for DNA repair[42,43]. We have already discussed that caffeine induces the metabolism of dacarbazine, but caffeine has another well stablished mechanism, the ability to inhibit DNA repair[44].In the absence of the possibility of DNA repair, p53 induces apoptosis, modifying the expressions of bax and bcl-2[45]. This mechanism collaborates with the explanation of increased apoptotic cells in association with caffeine, ande the involvement of p53 in apoptosis induction was confirmed by the increase in immunocytochemical staining of p53 (Fig. 6D). Previously, other authors also reported increased p53 levels in epidermal cells JB6 CL41 treated with caffeine[45].

Other cellular process was observed autophagy (Fig. 6A). Autophagy is a pathophysiological process involved in the renovation of damaged organelles and is related to apoptosis[46].Up to date, the only mechanism suggested to caffeine-induced autophagy is the inhibition of the PI3K / Akt / mTOR / p70S6K[47].

For *in vitro* model, we observed that animals implanted with B16F10 reduced the number of lung tumour nodules (29.2% reduction). Gude*et al.*[9], using the same experimental design of our study, also observed a reduction in the count of metastatic nodules in the caffeine treatment (50 and 100 mg/kg- 10 days), the authors proposed that caffeine (and methylxanthine derivatives) are capable of inhibit phosphodiesterase, an enzyme responsible

for degradation of cAMP, resulting in a decrease in adhesion and inhibition of metalloproteinases and angiogenesis.

We also observed a reduction of 71.3% in the counting of metastatic nodules in the group treated with DTIC (40 mg/kg, i.p.) associated with caffeine, a significant reduction compared to the group treated with DTIC (53.8%). The potentialization of antineoplastic agent by caffeine treatment, was also reported by other authors such as Kawahara et al[48].These authors reported the potentialization of cisplatin in animals with pulmonary metastases by association with caffeine.

Many of the mechanisms that we observed *in vitro*, such inhibition of cell cycle, increased genotoxicity and cytotoxicity of DTIC, generation of oxidative stress, induction of apoptosis and autophagy may be involved in the potentialization of DTIC *in vivo*. One important mechanism is the inhibition of nuclear translocation of ERK1/2, once this protein has multiple target genes, including VEGF, which the protein essential for vascularization process[49].

5. CONCLUSIONS

Our results indicate that adjuvant treatment with caffeine can be an alternative to improve the effectiveness of melanoma chemotherapy, being necessary to carry out new experiments *in vitro* and *in vivo* to elucidate more mechanisms involved and the safety of this association.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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LEGENDS OF FIGURES

Figure 1: Caffeine potentiation effect (concentrations 5, 10 and 20 µM- 24 hours) on B16F10 cells treated with dacarbazine (250 or 500µM- 4 hours). A) Cell viabillity results (MTT assay) obtained from post-treatment protocol,cells were exposed to dacarbazine (4h)prior to caffeine (24h). B) Cell viability, number of viable cells (trypan blue exclusion assay) and cell proliferation (cell counting) obtained from pre-treatment protocol, cells were exposed to tocaffeine (24h) prior to dacarbazine (4h). DTIC = dacarbazine, CAF = caffeine. [a]Significant difference compared to controlp> 0.05; [b]Significant difference compared to the control dacarbazine 250µM p> 0.05.[c]Significant difference compared to control dacarbazine 500µM.

Figure 2: Cell viability (MTT assay), number of viable cells (trypan blue exclusion assay) and cell proliferation (cell counting) of B16F10 cells treated with caffeine (24h). DTIC = dacarbazine, CAF = caffeine. [a]Significant difference compared to controlp> 0.05;

Figure 3: The figure show the results obtained from Comet assay for cells exposed to caffeine, DTIC or pre-treatment protocol (caffeine-24h prior to dacarbazine-4h). A) Induction of DNA damage (Comet assay)in B16F10 cells. B) Tables show the model analysis and variance analysis of tail moment results, and the responsesurface 3D ghaph shows the interaction between caffeine and dacarbazine treatment in the generation of DNA damage. DTIC= dacarbazine, CAF= caffeine. [a]Significant difference compared to controlp> 0.05; [b]Significant difference compared to the control dacarbazine 250µM p> 0.05. [c]Significant difference compared to control dacarbazine 500µM p> 0.05.

Figure 4: The figure show the results obtained from cell death pattern for B16F10 cells exposed to caffeine, DTIC or pre-treatment protocol (caffeine-24h prior to dacarbazine-4h). A) Lactase dehidrogenase (LDH) release into the medium revealing necrosis. B) TUNEL

assay revealing the percentage of apoptotic cells. DTIC= dacarbazine, CAF= caffeine. [a]Significant difference compared to controlp> 0.05; [b]Significant difference compared to the control dacarbazine 250µM p> 0.05. [c]Significant difference compared to control dacarbazine 500µM.

Figure 5: A) Results obtained from dacarbazine levels measure in cell culture of B16F10 cellsexposed todacarbazine or pre-treatment protocol (caffeine-24h prior to dacarbazine-4h) using a HPLC method (DTIC retention time = 3,2min, shown in HPLC chromatogram). B) Measument of malondealdehydelevels (nM). DTIC= dacarbazine, CAF= caffeine. [a]Significant difference compared to controlp> 0.05; [b]Significant difference compared to the control dacarbazine 250µM.

Figure 6: Results obtained from B16F10 cells treated with caffeine (24h). A) Percentage of cells with autophagic vacuoles revealed by monodansyldacaverine assay. B) ERK 1/2 labelled area/cell and percentage of cells with nuclear labelling for this protein. C) AKT labelled area/cell. D) Percentage of cell with nuclear p53 labeling. E) KI67 labelled area/cell. Images are representative photomichographs for each protein. CAF= caffeine. *Statistically different from control group (p<0,05).

Figure 7:Representative images of lungs from each treatment group.

TABLES

Table 1: Animal weight variation, number of pulmonary metastatic nodules and reduction in the percentage of lung nodules after treatment with oral caffeine (0,44mg/ml), and dacarbazine (40 mg/kg).

Group of treatment (number of animals)	Animal weight variation ±SE (g)	Number of lung tumour nodules	Reduction of lung tumour nodules compared to melanoma group (%)
Control (8)	0.6±0.1	0±0	-
Melanoma (6)	-5.7±0.5 [a]	128.0±3.0[b]	0
Caffeine (7)	-2.9±0.7 [a, b]	90.6±18.2[b]	29.2
Dacarbazine (6)	-2.7±0.7 [a, b]	63.6±10.7[b]	53.8
Pre-treatment (7)	-3.1±0.5 [a, b]	56.8±1.3[b, c, d]	71.3

[a]Significant difference compared to the control group p>0.05; [b]Significant difference compared to the melanoma group p> 0.05. [c]Significant difference compared to caffeine group p> 0.05. [d]Significant difference compared to dacarbazine group p> 0.05. The percentage of nodules reduction was calculate by the formula: Reduction = 100%- (Number of tumour nodules of test group *100/ Number of tumor nodules of melanoma group).

FIGURES

Figure 1

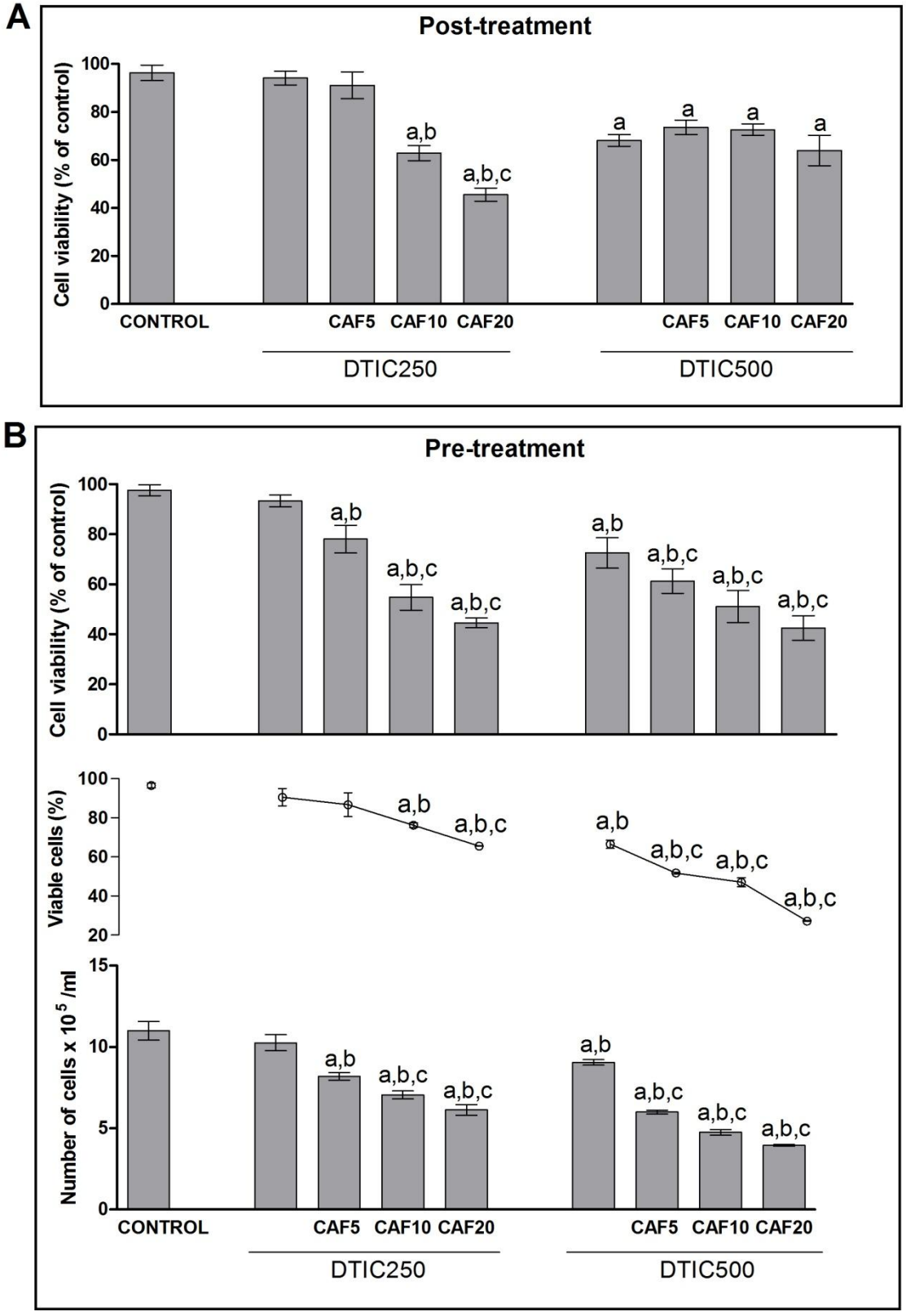


Figure 2

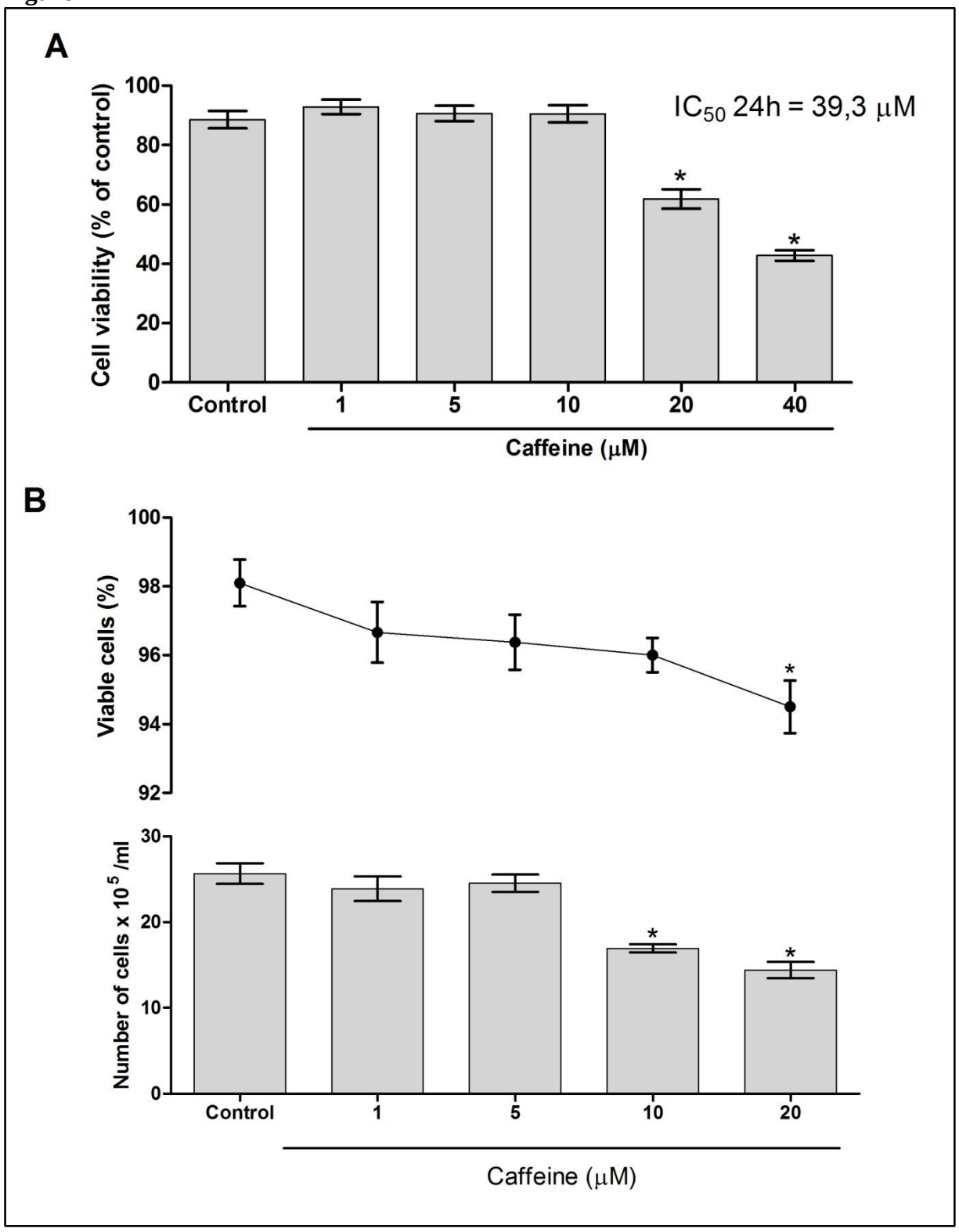
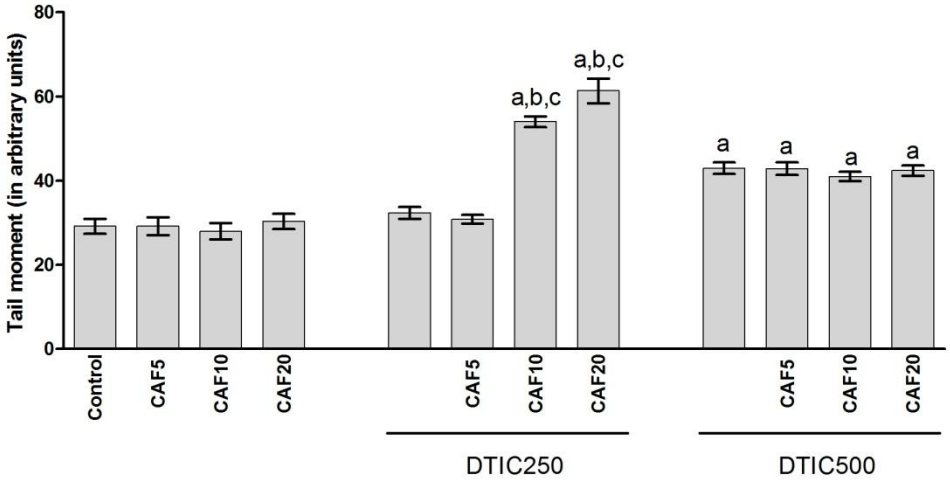

A
Cell viability (% of control)
100
80
60
40
20
0
IC50 24h = 39,3 μM
*
*
Control
1
5
10
20
40
Caffeine (μM)
B
Viable cells (%)
100
98
96
94
92
*
Number of cells x 10^5 /ml
30
20
10
0
*
*
Control
1
5
10
20
Caffeine (μM)

Figure 3

A



B

Model analysis

Factor	Effect Estimates; Var.:COMET ASSAY; R-sqr=,91319; Adj:,90505 (Design: 2**(2-0) design (Spreadsheet16) in Workbook1) 2**(2-0) design; MS Residual=7,381944									
	Effect	Std.Err.	t(32)	p	-95,% Cnf.Limt	+95,% Cnf.Limt	Coeff.	Std.Err. Coeff.	-95,% Cnf.Limt	+95,% Cnf.Limt
Mean/Interc.	**42.72**	**0.45**	**94.34**	**0.00**	**41.80**	**43.65**	**42.72**	**0.45**	**41.80**	**43.65**
(1)[CAF]	**11.22**	**0.91**	**12.40**	**0.00**	**9.38**	**13.07**	**5.6**	**0.45**	**4.69**	**6.53**
(2)[DTIC]	-0.89	0.91	-0.98	0.33	-2.73	0.96	-0.44	0.45	-1.37	0.48
1 by 2	**-12.22**	**0.91**	**-13.50**	**0.00**	**-14.07**	**-10.38**	**-6.11**	**0.45**	**-7.03**	**-5.19**

Variance analysis

Factor	ANOVA; Var.:COMET ASSAY; R-sqr=0.91319; Adj:0.90505 (Design: 2**(2-0) design (Plan1 in Workbook1_(Recovered)) in Workbook2_1) 2**(2-0) design; MS Residual=7.381944 DV: COMET				
	SS	df	MS	F	p
(1)[CAF]	**1133.44**	**1**	**1133.44**	**153.54**	**0.00**
(2)[DTIC]	7.11	1	7.11	0.96	0.33
1 by 2	**1344.44**	**1**	**1344.44**	**182.13**	**0.00**
Error	236.22	32	7.38		
Total SS	2721.22	35			

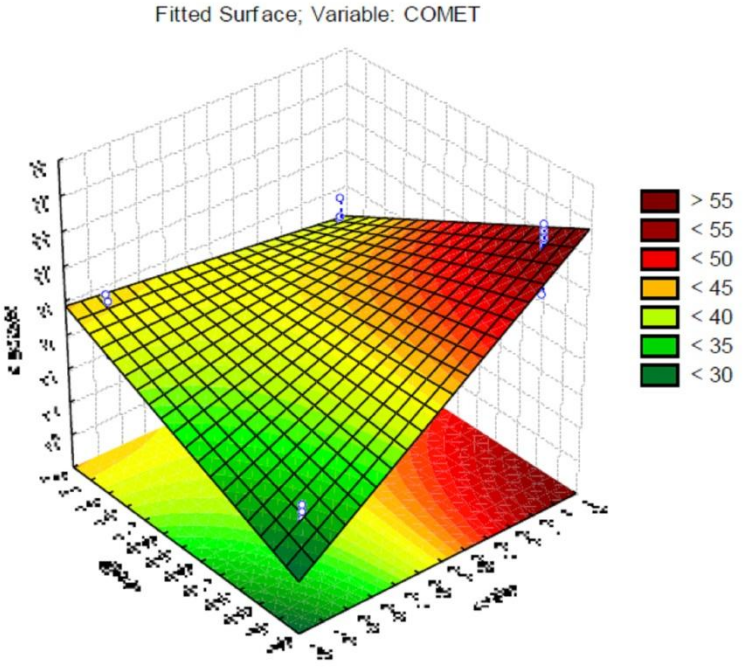


Figure 4

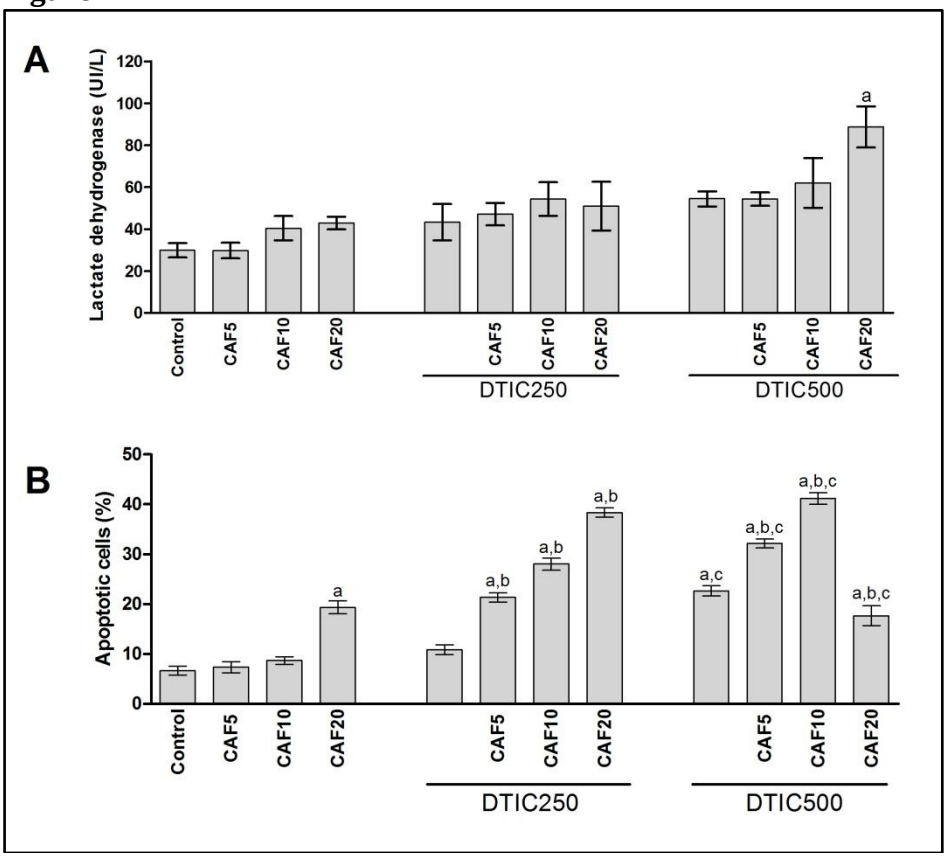


Figure 5

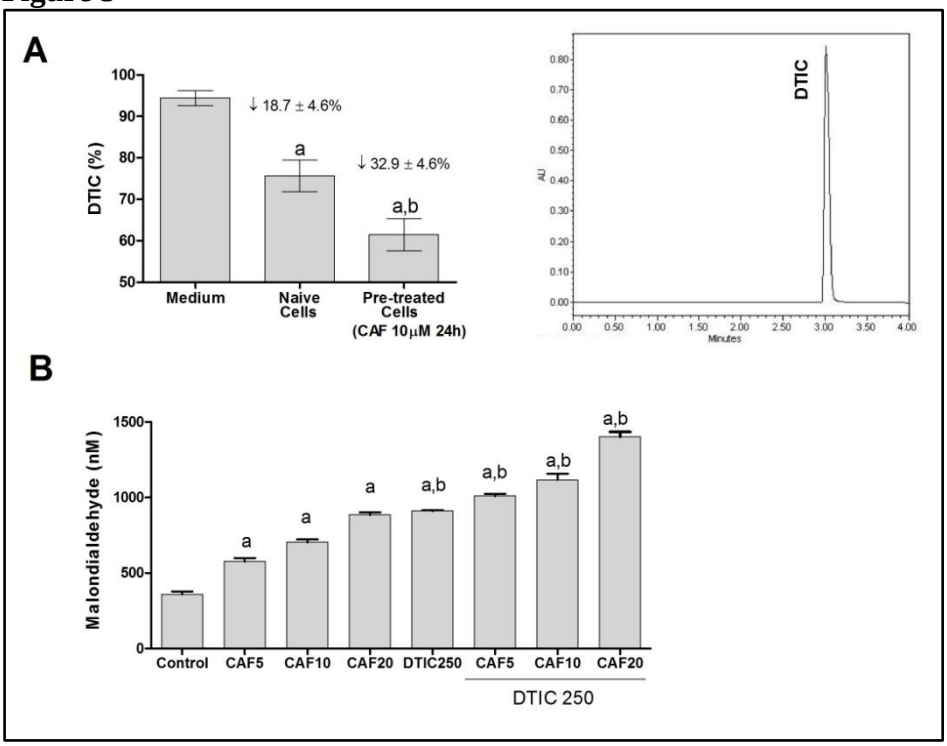


Figure 6

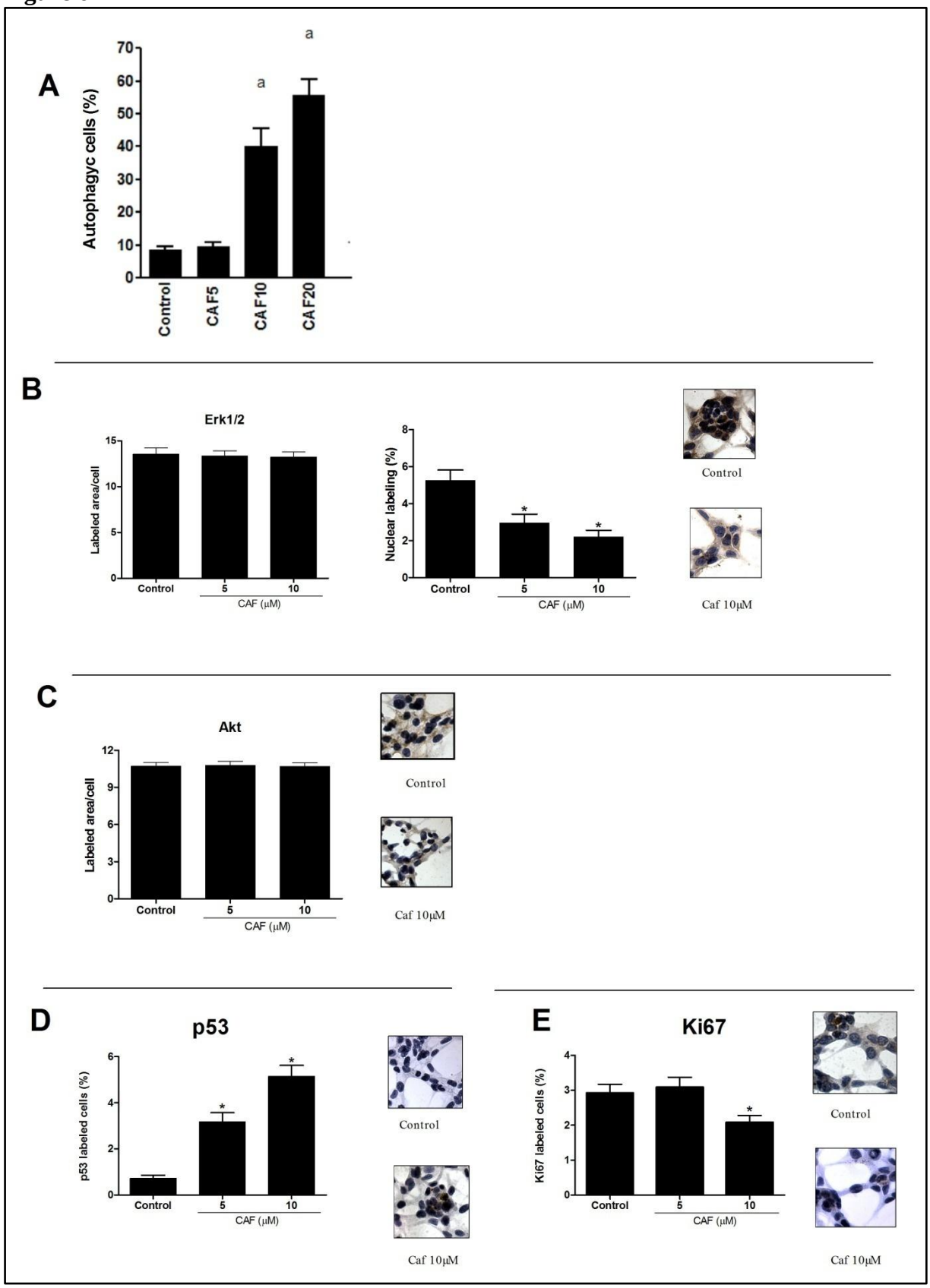
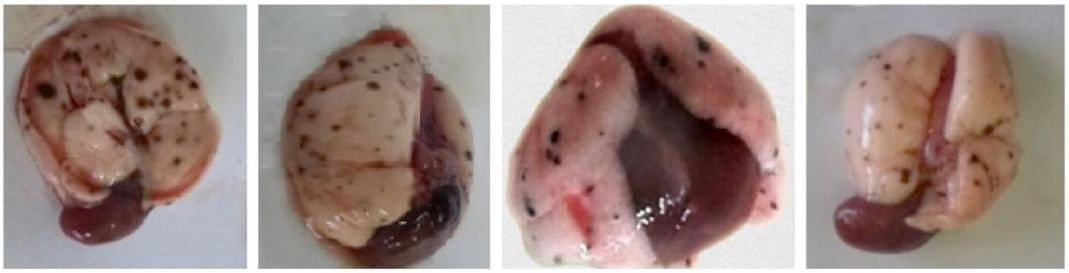

A
Autophagyc cells (%)
a
a
Control
CAF5
CAF10
CAF20
B
Erk1/2
Labeled area/cell
Control
5
10
CAF (μM)
Nuclear labeling (%)
Control
Caf 10μM
C
Akt
Labeled area/cell
Control
Caf 10μM
D
p53
p53 labeled cells (%)
Control
Caf 10μM
E
Ki67
Ki67 labeled cells (%)
Control
Caf 10μM

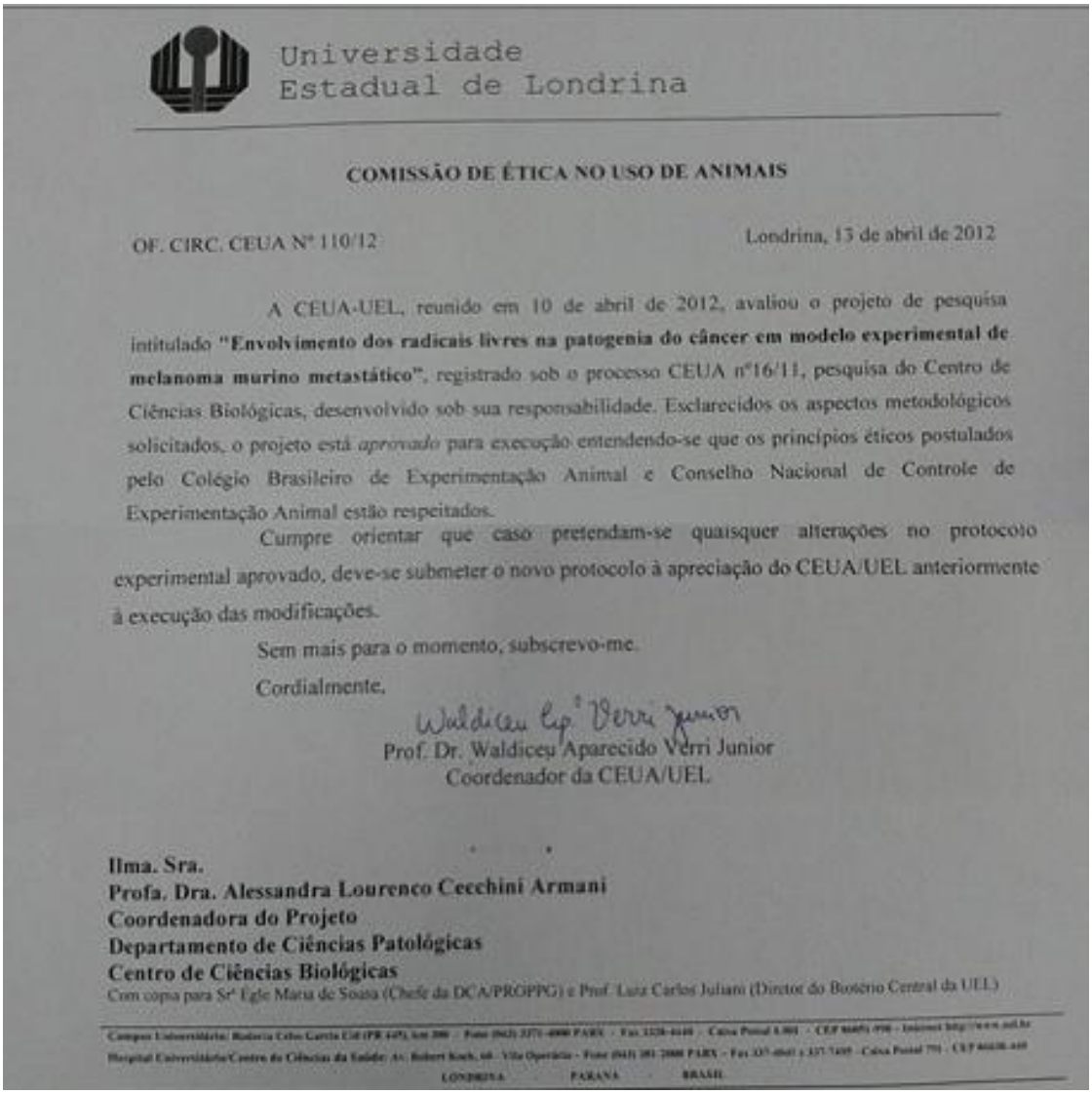
Figure 7



Melanoma Caffeine Dacarbazine Pre-treatment

Anexo B

Parecer de aprovação do Comitê de Ética em Pesquisa Envolvendo Animais da Universidade Estadual de Londrina.



Universidade
Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 110/12 — Londrina, 13 de abril de 2012

A CEUA-UEL, reunido em 10 de abril de 2012, avaliou o projeto de pesquisa intitulado **"Envolvimento dos radicais livres na patogenia do câncer em modelo experimental de melanoma murino metastático"**, registrado sob o processo CEUA nº16/11, pesquisa do Centro de Ciências Biológicas, desenvolvido sob sua responsabilidade. Esclarecidos os aspectos metodológicos solicitados, o projeto está *aprovado* para execução entendendo-se que os princípios éticos postulados pelo Colégio Brasileiro de Experimentação Animal e Conselho Nacional de Controle de Experimentação Animal estão respeitados.

Cumpre orientar que caso pretendam-se quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação do CEUA/UEL anteriormente à execução das modificações.

Sem mais para o momento, subscrevo-me.

Cordialmente,

Waldiceu Aparecido Verri Junior

Prof. Dr. Waldiceu Aparecido Verri Junior
Coordenador da CEUA/UEL

Ilma. Sra.
Profa. Dra. Alessandra Lourenco Cecchini Armani
Coordenadora do Projeto
Departamento de Ciências Patológicas
Centro de Ciências Biológicas
Com cópia para Srª Egle Maria de Souza (Chefe da DCA/PROPPG) e Prof. Luiz Carlos Juliani (Diretor do Biotério Central da UEL)

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Hospital Universitário/Centro de Ciências da Saúde: Av. Robert Koch, 60 - Vila Operária - Fone (043) 3371-2000 PABX - Fax 337-4641 e 337-7409 - Caixa Postal 791 - CEP 86038-440
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