UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA
CAMPUS DE BOTUCATU

EFEITO DA PRÓPOLIS SOBRE A PRODUÇÃO DE CITOCINAS POR CAMUNDONGOS SUBMETIDOS A ESTRESSE CRÔNICO E DESAFIADOS COM MELANOMA

FABIANE MISSIMA

Tese apresentada ao Programa de Pós-Graduação em Patologia da Faculdade de Medicina de Botucatu, Universidade Estadual Paulista – UNESP para obtenção do título de Doutor.

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"Cada homem tem seu lugar no mundo e no tempo que lhe é concedido. Sua tarefa nunca é maior que sua capacidade para poder cumpri-la. Ela consiste em preencher seu lugar, em servir a verdade e aos homens."

(João Guimarães Rosa)

SUMÁRIO

Revisão Bibliográfica

1. Revisão Bibliográfica

Em virtude de suas inúmeras propriedades biológicas, dentre elas, a imunomoduladora (SFORCIN, 2007), a própolis foi empiricamente utilizada por séculos.

A própolis é um produto natural, elaborado pelas abelhas a partir de material coletado em botões, gemas e córtex vegetais, sendo um conjunto complexo de substâncias (BANKOVA, 2005). A própolis apresenta, de forma genérica, em sua composição química *in natura* 55% de resinas e bálsamos, 30% de cera, 10% de óleos voláteis e 5% de pólen, além de impurezas mecânicas. GREENAWAY *et al.* (1991) identificaram as estruturas químicas de muitos componentes da própolis: flavonóides, cumarinas, aldeídos aromáticos, ácidos aromáticos, ácidos orgânicos, minerais, vitaminas, aminoácidos, polissacarídeos. O método de preparo dos extratos de própolis pode influenciar sua atividade, uma vez que os diferentes solventes solubilizam e extraem compostos diferentes. Os extratos mais utilizados experimentalmente são os etanólicos, em diferentes concentrações, o metanólico e o aquoso (CUNHA *et al.*, 2004).

A composição química da própolis por nós coletada ao longo das quatro estações na região de Botucatu, SP, foi analisada, verificando-se que diferenças sazonais não são significantes, mas predominantemente quantitativas (BOUDOUROVA-KRASTEVA *et al.*, 1997; BANKOVA *et al.*, 1998). Ademais, as fontes vegetais da própolis obtida no apiário de nossa Universidade foram investigadas, sendo *Baccharis dracunculifolia* a principal fonte, seguida de *Araucaria angustifolia* e *Eucalyptus citriodora* (BANKOVA *et al.*, 1999).

SFORCIN *et al.* (2002b) demonstraram que a administração de própolis a ratos, 2 vezes ao dia, durante 3 dias consecutivos, não induziu alterações na concentração sérica de componentes bioquímicos de significado clínico. Neste trabalho, foi observado também a ausência de efeito da sazonalidade sobre a atividade da própolis em variáveis bioquímica. Dando continuidade a este trabalho, MANI *et al.* (2006) avaliaram o efeito de diferentes concentrações de própolis administradas a ratos, o efeito de diferentes extratos (aquoso e etanólico), e o efeito do tratamento a longo prazo (30, 90 e 150 dias) sobre a concentração sérica de variáveis bioquímicas. Os resultados não evidenciaram diferenças entre o grupo controle e os grupos tratados com própolis, sugerindo a ausência de efeitos colaterais após tratamento com este produto apícola.

Em estudos toxicológicos *in vitro,* TAVARES *et al.* (2006) sugeriram que baixa concentração de própolis (12.5 µg/mL) apresenta efeito antimutagênico, enquanto que alta concentração (100 µg/mL) parece ser mutagênica. PEREIRA *et al*. (2008) avaliaram o efeito mutagênico da própolis *in vivo* em eritrócitos de camundongos tratados com 1000, 1500 e 2000 mg/Kg, concluindo que estas concentrações podem acarretar efeitos mutagênicos.

Atualmente, a própolis tem despertado a atenção de pesquisadores, no intuito de elucidar cientificamente suas propriedades biológicas (BANSKOTA *et al*., 2001). Nesse sentido, a ação antibiótica da própolis tem sido investigada por nosso grupo, evidenciando-se sua ação antibacteriana, antiviral, antifúngica e anti-parasitária (SFORCIN *et al.*, 2000; SFORCIN *et al.*, 2001; BÚFALO, 2006; FREITAS *et al*., 2006; ORSI *et al*., 2006).

Com relação ao sistema imune e seus mecanismos efetores, SCHELLER *et al*. (1988) verificaram que a própolis estimula a formação de anticorpos por células esplênicas de camundongos BALB/c imunizados com hemácias de carneiro. Avaliando

o efeito deste apiterápico sobre a ativação de células *natural killer* (NK) contra células tumorais, observamos que a administração de própolis a ratos, durante 3 dias, induziu aumento na atividade lítica de células NK, quando comparada com o do grupo controle (SFORCIN *et al.*, 2002a). O tratamento com própolis durante 3 dias também induziu aumento na produção de anticorpos em ratos imunizados com albumina sérica bovina (SFORCIN *et al.*, 2005). Estes achados reforçam a afirmação prévia de SCHELLER *et al.* (1988), os quais sugeriram que este apiterápico atua sobre o sistema imune em curto prazo, após sua administração, e que a atividade imunoestimulante da própolis pode estar associada com a ativação de macrófagos e aumento de sua capacidade fagocítica. TATEFUJI *et al.* (1996) estudaram o efeito de seis componentes presentes na própolis e observaram que, dentro das propriedades biológicas apresentadas por este apiterápico, houve um aumento no espraiamento de macrófagos e na sua mobilidade.

Trabalhos experimentais de nosso laboratório revelaram também que macrófagos peritoneais de camundongos estimulados com própolis apresentaram aumento na atividade fungicida contra *Paracoccidioides brasiliensis* (MURAD *et al.*, 2002) e na atividade bactericida contra *Salmonella* Typhimurium (ORSI *et al.*, 2005). Foi evidenciado também que a própolis induz elevação na geração de peróxido de hidrogênio (H_2O_2) por macrófagos peritoneais de camundongos e inibição na liberação de óxido nítrico (NO) por estas células, de forma dose-dependente (ORSI *et al.*, 2000).

LOPES *et al.* (2003) avaliaram a ação de extratos das plantas fontes de própolis em nossa região sobre a produção de H_2O_2 e NO por macrófagos, verificando a ausência de efeito sobre a produção de tais metabólitos. Estes resultados sugeriram que a ação da própolis é uma conseqüência dos produtos derivados das plantas e adição de substâncias secretadas pelas abelhas, atuando possivelmente de forma sinérgica. Considerando que *Baccharis dracunculifolia* DC é a principal fonte de própolis em

nosso apiário e dando continuidade a estes ensaios, avaliamos o efeito do extrato total das partes aéreas, extrato do lavado glandular, extrato das folhas, extrato das raízes e óleo essencial desta planta sobre o estado de ativação de macrófagos, bem como de substâncias purificadas. Os dados revelaram que o extrato do lavado glandular, extrato das folhas e extrato das raízes induziram elevação na geração de H_2O_2 por macrófagos. Com relação aos compostos isolados, o óxido de *Baccharis* e o friedelanol estimularam a produção de H_2O_2. Estes resultados sugerem uma ação ativadora de extratos e compostos isolados de *B. dracunculifolia* sobre macrófagos (MISSIMA *et al*., 2007).

Vários pesquisadores têm relatado a propriedade antitumoral da própolis (CHIAO *et al.*, 1995; RAO *et al.*, 1995). Trabalhos de nosso grupo evidenciaram que o extrato hidroalcoólico de própolis possui papel protetor no processo de carcinogênese do cólon, suprimindo o desenvolvimento de lesões pré-neoplásicas (BAZO *et al.*, 2002). Utilizando extrato aquoso de própolis, verificamos que este reduziu os danos induzidos pela dimetilhidrazina no DNA de células do cólon de ratos (ALVES DE LIMA *et al.*, 2005). Em ensaios *in vitro*, verificamos sua eficiente ação citotóxica contra células de tumor venéreo transmissível de cães (BASSANI-SILVA *et al.*, no prelo) e contra células de carcinoma epidermóide de laringe (BÚFALO *et al*., no prelo), de forma dose- e tempo-dependente.

ORSOLIC *et al.* (2004), avaliando o efeito da própolis, bem como de compostos polifenólicos isolados da mesma, sobre o crescimento e potencial metastático de carcinoma mamário transplantável em camundongos, relataram que a atividade antitumoral está associada à propriedade imunomoduladora dos compostos, à citotoxicidade em relação às células tumorais, indução de apoptose e necrose.

Em virtude de sua ação imunomoduladora, ensaios têm sido desenvolvidos em nossos laboratórios para investigar o papel da própolis durante o estresse. O conceito de

estresse foi apresentado por Hans Selye em 1936, tendo havido, posteriormente, um crescente interesse dos pesquisadores quanto à identificação dos agentes estressores bem como dos eventos fisiológicos envolvidos na resposta ao estresse.

O estresse é comumente definido como uma condição ou estado em que a homeostase do organismo é perturbada, como resultado de estímulos estressores. O estresse é uma constelação de eventos, envolvendo a participação de diferentes sistemas do organismo em resposta a agentes estressores, como fatores climáticos, hiperpopulação, infecções, exercício físico intenso, desnutrição, ruído, odor, entre muitos outros (KIOUKIA-FOUGIA *et al.*, 2002).

A crescente complexidade social também tem contribuído com o aparecimento de tensão psicológica e doenças relacionadas ao estresse, como câncer, depressão, ansiedade, pânico, dermatite atópica, colite ulcerativa, entre outras. Dentre os agentes estressogênicos da vida moderna, temos a competição profissional, desemprego, violência, desagregação familiar, relações interpessoais instáveis, solidão, preocupação financeira persistente, pressão da globalização e tecnologia, entre outras causas de maior impacto, como morte, doença grave, acidentes, entre outros (OLFF, 1999).

Há três fases importantes junto à “Síndrome Geral de Adaptação” ao estresse: a reação de alarme, na qual o organismo percebe o estímulo estressante; o estágio de resistência - uma tentativa de adaptação do organismo frente ao agente estressor; e fase de exaustão, quando o organismo perde a capacidade de adaptação, aparecendo lesões ou doenças (SELYE, 1978).

A resposta ao estresse envolve a ativação do sistema nervoso autônomo (SNA), bem como a ativação do eixo hipotálamo-pituitária-adrenal (HPA), com conseqüente liberação de catecolaminas e glicocorticóides, respectivamente. Em resposta à estimulação simpática, a epinefrina e norepinefrina são rapidamente secretadas para a

corrente sanguínea como uma resposta adaptativa, capacitando o organismo a responder à condição estressante, apresentando efeito pronunciado no sistema cardioavascular e na mobilização nas reservas de energia (LUNDBERG, 2005).

O estresse origina impulsos nervosos, que são transmitidos desde a periferia até o hipotálamo. O hipotálamo, então, secreta o fator liberador de corticotropina (CRH), que passa pelo sistema porta hipotálamo-hipofisário chegando até a hipófise anterior. Neste local, o CRH induz a secreção de hormônio adrenocorticotrópico (ACTH), que flui pelo sangue até o córtex da adrenal, induzindo a secreção de glicocorticóides, como cortisol, corticosterona e aldosterona (SELYE, 1978).

O estresse é uma resposta adaptativa fundamental à sobrevivência, presente não só em mamíferos, mas também em outros vertebrados. Os mecanismos básicos e moléculas envolvidas na resposta ao estresse são similares e bem preservados ao longo da evolução das espécies (OTTAVIANI & FRANCESCHI, 1996).

O estresse agudo pode ser entendido como uma ameaça imediata, a curto prazo, comumente conhecido como resposta à luta ou fuga. A “ameaça” pode ser qualquer situação experienciada como perigo, e, após o evento, que dura minutos a horas, há uma resposta de relaxamento (DHABHAR, 2002). Por outro lado, o estresse é denominado crônico quando persiste por várias horas a dias, semanas ou meses. Como exemplo, temos modelos experimentais com imobilização, exposição à temperatura adversa, exercício físico intenso, entre outros. Modelos experimentais de estresse crônico são apropriados para a investigação da relação estresse-depressão (BAUER *et al.*, 2000).

O modelo de imobilização é um procedimento que não causa dor e o animal não é comprimido fisicamente, mas a sensação de confinamento induz estresse psicológico, afetando o SNA e o eixo HPA, resultando em níveis elevados de catecolaminas e corticosterona (BAUER *et al.*, 2001).

Há vários indicadores de estresse encontrados na literatura pertinente, como concentração de corticosterona, glicemia, atividade da desidrogenase láctica (LDH), aparecimento de úlcera, determinação de hormônio adrenocorticotrófico (ACTH), entre outros. A determinação de cortisol é utilizada como indicador de estresse em seres humanos, e a de corticosterona em roedores (BESEDOVSKY & DEL REY, 1996; LUNDBERG, 2005).

Muitas evidências têm sugerido que o estresse modula a resposta imune (BESEDOVSKY & DEL REY, 1996). A Imunoneuroendocrinologia ou Psiconeuroimunologia tem despertado a atenção dos pesquisadores quanto ao estudo da relação entre os sistemas imune, nervoso e endócrino, os quais compartilham receptores para citocinas, neurotransmissores e hormônios. O comportamento doentio reflete esta íntima relação, visto que citocinas podem diminuir a atividade geral do organismo, afetar o comportamento social e sexual, e diminuir a atividade exploratória do indivíduo. Como exemplo, a interleucina-1 (IL-1) é considerada um pirógeno endógeno, induzindo febre e alteração comportamental (PARMET *et al.*, 2002).

Com relação ao sistema imune, os distúrbios resultantes do estresse podem variar de acordo com o tipo, intensidade e duração do agente estressor. Da mesma forma, diferenças quanto à linhagem, idade e sexo dos animais de experimentação, bem como o momento de mensuração de determinados parâmetros, também podem afetar a avaliação da resposta imunológica (KIOUKIA-FOUGIA *et al.*, 2002).

O estresse crônico pode induzir imunossupressão (ELENKOV & CHROUSOS, 1999). Glicocorticóides são considerados imunossupressores, sendo clinicamente utilizados como agente anti-inflamatório. Como linfócitos, monócitos, macrófagos e granulócitos possuem receptores para vários mediadores do SNA e do eixo HPA, como catecolaminas e corticóides (PADGETT & GLASER, 2003), observa-se, no estresse

crônico, inibição na síntese de prostaglandinas, na produção de citocinas e proliferação de linfócitos (DHABHAR, 2002). A produção de IL-3, uma das citocinas fundamentais para hematopoiese, também encontra-se inibida em resposta ao estresse e em indivíduos com depressão, sugerindo a interligação entre sistema nervoso e sistema imune (BESSLER *et al.*, 2000). DOMÍNGUEZ-GERPE & REY-MÉNDEZ (2001) relataram alterações em subpopulações de linfócitos após estresse crônico por imobilização, demonstrando que células T foram mais afetadas que células B.

A resposta imune celular é mais afetada durante o estresse, em relação à imunidade humoral. O estresse pode afetar o padrão Th1/Th2 de resposta, direcionando-o mais para Th2, podendo suprimir a imunidade celular e reforçar a imunidade humoral, por ação de glicocorticóides e catecolaminas (IWAKABE *et al.*, 1998; ELENKOV & CHROUSOS, 1999; ELENKOV, 2004).

A imunossupressão causada pelo estresse pode afetar a metástase de tumores e promover a progressão do câncer (BEN-ELIYAHU, 2003).

O câncer de pele é a terceira neoplasia mais comum a acometer o homem, e sua incidência global tem sido progressiva. As formas mais comuns de neoplasias de pele são os carcinomas de células basais, os carcinomas de células escamosas e os melanomas – a forma mais perigosa e letal. Quando diagnosticado na fase inicial, 80% dos casos de melanomas são curados através de remoção cirúrgica, mas sua forma metastática é altamente refratária às terapias existentes (GRAY-SCHOPFER *et al.*, 2007).

Considerando que o estresse pode induzir imunossupressão e, conseqüentemente, o desenvolvimento de tumores, introduzimos, em nosso modelo experimental, o tratamento dos animais com própolis, visto que este produto apícola apresenta propriedades imunomoduladora e antitumoral. Ademais, poucos são os

trabalhos encontrados na literatura sobre os efeitos da própolis no sistema imunológico, principalmente quanto à determinação de citocinas.

Assim, nosso trabalho visou contribuir quanto ao efeito da própolis sobre a produção de citocinas pró-inflamatórias e de padrão Th1/Th2, bem como sobre o desenvolvimento tumoral *in vivo*, pois há inúmeros trabalhos sobre a ação antitumoral da própolis, sendo a maioria deles, porém, realizada com ensaios *in vitro*.

2. Objetivos

- Avaliar o efeito da própolis sobre a produção de citocinas pró-inflamatórias (IL-1β e IL-6), de padrão Th1 (IL-2 e IFN-γ) e Th2 (IL-4 e IL-10) através da técnica de ELISA;
- Observar o possível papel imunorestaurador da própolis quanto à produção destas citocinas em animais estressados;
- Quantificar a expressão gênica das citocinas de padrão Th1 e Th2 produzidas por esplenócitos, pela técnica de PCR em tempo real;
- Observar a ação imunomoduladora da própolis em camundongos estressados, portadores ou não de melanoma;
- Avaliar os níveis séricos de corticosterona dos animais.

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*Manuscrito 1**

* De acordo com as normas da revista Brain, Behavior, and Immunity. As normas constam no anexo 1 deste trabalho (página 63).

Propolis effect on Th1/Th2 cytokines expression and production by melanoma-bearing mice submitted to stress

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Abstract

Several studies have shown that stress affects the immune system, promoting the progression of cancer. Since propolis is a honeybee product showing immunomodulatory and antitumoral activities, the goal of this work was to evaluate propolis effect on Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokines' expression and production by melanoma-bearing mice submitted to immobilization stress. In groups without melanoma, stress did not affect IL-2, IL-4 and IL-10 gene expression. Propolis treatment of stressed animals did not influence these cytokines expression as well. On the other hand, IL-2 and IL-10 expression was inhibited in melanoma-bearing mice, stressed or not. Th1 cytokines production was also inhibited in melanoma-bearing mice. Propolis administration to melanoma-bearing mice submitted to stress stimulated IL-2 expression, as well as Th1 (IL-2 and IFN-γ) production, indicating the activation of antitumor cell-mediated immunity. Propolis also stimulated IL-10 expression and production, what may be related to immunoregulatory effects. Our data indicate that propolis showed an immunomodulatory activity in this assay, what may be related to its antitumoral action in vivo. Further investigation will help to understand propolis usefulness during stress.

Keywords: Stress; Melanoma; Cytokines; Propolis; Immunomodulation

1. Introduction

Stress comprises several events, activating physiologic systems in the body to cope with perceived stimuli, and altering the homeostatic state of the organism. Evidences have shown a communication between the immune, endocrine and central nervous systems during stress, resulting in behavioral, endocrine and immunological changes. Stress responses induce activation of both autonomic nervous system and hypothalamic-pituitary-adrenal (HPA) axis, with consequent release of catecholamines and glucocorticoids, respectively (Bauer et al., 2001; Dhabhar, 2002).

Viveros-Paredes et al. (2006) reported that glucocorticoids inhibit the production of IL-2 and IFN-γ by Th1 cells, up-regulate the production of IL-4, IL-10 and IL-13 by Th2 cells, and increase the production of TGF-β by T cells, suppressing cellular immunity and stimulating humoral immunity. Because of glucocorticoids' immunosuppressive effects, stress plays a role in the etiology of many diseases, such as cancer, being detrimental to health.

Melanoma is among the most immunogenic of all solid cancers, and the presence of tumor antigen-specific antibodies and tumor-specific cytotoxic T cells in the peripheral blood of melanoma patients has been well established (Fang et al., 2008). B16F10 is a selective variant cell line obtained from pulmonary metastasis of a melanoma, syngeneic to black C57BL/6 mice (Sá-Rocha et al., 2006). While stress does not seem to affect humoral immunity, a Th2 cytokine profile is associated with down-regulation of the immune response and progression of melanoma (McCarter et al., 2005).

The interaction between stress and immunity has been well documented, but the use of natural products during stress deserves investigation, mainly in melanoma-bearing mice. Propolis is a resinous hive product showing several therapeutic activities,

including immunomodulatory and antitumoral. In vitro and in vivo assays demonstrated the modulatory action of propolis on murine peritoneal macrophages, increasing their microbicidal activity. Its stimulant action on the lytic activity of natural killer cells against tumor cells, and on antibody production was demonstrated (Sforcin, 2007). The antitumoral property of propolis has been investigated both in vivo and in vitro (Sforcin et al., 2002; Búfalo et al., in press).

Based on these observations, the aim of this work was to analyze propolis effects on Th1/Th2 cytokines' expression and production by melanoma-bearing mice submitted to chronic stress, in order to investigate a possible immunorestorative action of propolis in these animals.

2. Methods

2.1. Animals

C57BL/6 male mice aged between 8 and 12 weeks were kept in rooms at 21-25 ºC, with a 12 h /12 h light/dark cycle. Food and water were provided *ad libitum*. This work agrees with Ethical Principles in Animal Research adopted by Brazilian College of Animal Experimentation (nº 464).

2.2. Melanoma cells

B16F10 cells were cultured in DMEM (Cultilab, Campinas, SP, Brazil), supplemented with 25 mM HEPES (Sigma – Aldrich, St. Louis, MO, USA) and 10% fetal calf serum. Cell suspensions were detached from the culture flasks using 0.2% trypsin (Nutricell, Campinas, SP, Brazil), and viable cells were counted using a

hemocytometer. Mice were inoculated with 5 x 10^4 cells in 0.1 mL of phosphate-buffered saline subcutaneously (s.c.) into the right flank region. Tumor development was monitored weekly.

2.3. Propolis sample

Propolis was collected in the Beekeeping Section, UNESP. Propolis was ground and 30% ethanolic extracts of propolis were prepared (30 g of propolis, completing the volume to 100 mL with 70% ethanol), in the absence of bright light, at room temperature, with moderate shaking. After a week, extracts were filtered and the dry weight of the extracts was calculated (120 mg/mL) (Búfalo et al., in press). Propolis chemical composition was investigated using thin-layer chromatography (TLC), gas-chromatography (GC), and gas chromatography-mass spectrometry (GC-MS) analysis (Bankova et al., 1998).

2.4. Experimental groups and stress procedure

Mice were divided into 8 groups (G1, G2, G3, G4, G5, G6, G7, G8), of 8 animals each, according to the experimental design:

	G1	G2	G3	G4	G5	G6	G7	G8
STRESS	-	+	-	+	-	+	-	+
PROPOLIS	-	-	+	+	-	-	+	+
MELANOMA	-	-	-	-	+	+	+	+

G1 was considered as control, and received physiologic solution (NaCl 0.9%). G2 was submitted to restraint stress in a well-ventilated immobilization tube (restrainer) of about 50 mL capacity for 15, 30, 45, 60, 75, 90 and 120 minutes during 7 consecutive days, and from 7^{th} to 14^{th} day, mice were submitted to restraint stress for 2 hours a day, at a fixed time between 8:00 and 11:00 a.m. This procedure is easily performed and causes no physical pain to the animals (Domínguez-Gerpe and Rey-Méndez, 2001; Sforcin et al., 2007).

G3 was treated daily with propolis (200 mg/kg in 0.1 mL, orally), and G4 was treated daily with propolis and submitted to the same stress protocol (Missima and Sforcin, 2008). After 24 h of the respective treatments, animals were sacrificed using a CO_2 inhalation chamber.

G5 was inoculated with B16F10 (5×10^4 cells) s.c. into the right flank. G6 was inoculated with B16F10 cells and submitted to stress. G7 was inoculated with B16F10 cells and treated daily with propolis. G8 was inoculated with B16F10 cells, treated daily with propolis and submitted to stress. All groups had no water and food during stress. After 14 days of melanoma inoculation, mice were sacrificed and metastases were investigated.

70% ethanol (propolis solvent) effects were also investigated after its administration for 14 days to mice.

2.5. Corticosterone determination

Before sacrifice, blood was collected by cardiac puncture and serum was stored at -20^{o} C. Corticosterone concentrations were determined by

radioimmunoassay, using a commercial kit (Coat-A-count, DPC, Los Angeles, CA, USA).

2.6. Spleen cells cultures and cytokines determination

After sacrifice, spleens were aseptically removed and cells were suspended at a concentration of 5 x 10^6/mL in RPMI 1640 (Cultilab, Campinas, SP, Brazil) supplemented with 10% fetal calf serum and 1% L-glutamine and cultured in flat-bottomed 24-well plates. Cells were cultured in triplicates (1 mL/well) and stimulated with concanavalin A (Con A – 5 μg/mL) for 48h and 5% CO_2.

Supernatants of spleen cell cultures were collected and assayed for Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokines determination by enzyme-linked immunosorbent assay (ELISA), according to manufacturer's instructions (BD Biosciences, San Diego, USA). Briefly, a 96-well flat bottom Maxisorp (Nunc, USA) was coated with capture antibody specific to each cytokine. The plate was washed and blocked before 100 μL of the supernatants and serially diluted specific standards were added to the respective wells. Following a series of washing, the captured cytokine was detected using the specific conjugated detection antibody. The chromogen/substrate reagent was added into each well and, after color development, the plate was read at 450 nm, using an ELISA plate reader (Tan et al., 2006).

2.7. Cytokines gene expression

2.7.1. mRNA extraction and cDNA synthesis

A portion of 30 mg of the spleens was kept in RNA safer (Omega Bio-tek, Inc. USA). Total RNA was extracted with RNAspin extraction kit (GE Healthcare UK

Limited) and further treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) for 30 min at 37 ºC to avoid false-positive results due to amplification of genomic DNA. cDNA was synthesized from 4 μg of total RNA in a final volume of 80 μl reaction using the Improm RT II (Promega, Madison, WI, USA), with random primers at 12.5 ng/μl following manufacturer's protocol.

2.7.2. Primers

Primers were designed based on sequences retrieved from Genebank using the Primer Express (Applied Biosystems, USA) and IDTSciTools (http://www.idtdna.com) softwares. For IL-2: primers forward 5'- CCC AAG CAG GCC ACA GAA TTG AAA-3' and reverse 5'- AGT CAA ATC CAG AAC ATG CCG CAG-3' (accession no. **X01772**); for IFN-γ: primers forward 5'- AGA GGA TGG TTT GCA TCT GGG TCA-3' and reverse 5'- ACA ACG CTA TGC AGC TTG TTC GTG-3' (accession no. **M28381**); for IL-4: primers forward 5'- AGA TGG ATG TGC CAA ACG TCC TCA-3' and reverse 5'- AAT ATG CGA AGC ACC TTG GAA GCC-3' (accession no. **NM_021283**); for IL-10: primers forward 5'- GGA CAA CAT ACT GCT AAC CGA C-3' and reverse 5'- TGG ATC ATT TCC GAT AAG GCT TG-3' (accession no. **M37897**). As an endogenous control, primers for β-actin found in literature were used (forward 5´-AAG TGT GAC GTT GAC ATC CGT AA- 3´ and reverse 5´-TGC CTG GGT ACA TGG TGG TA-3´) (Yang et al., 2006).

2.7.3. Real-time PCR

cDNA (4 μL) was amplified by real-time PCR with 2X Power Sybr® Green PCR Master Mix and 300 nM target primers. β-actin was used as the housekeeping gene. Each sample was analyzed in duplicates using SDS 7300 (Applied Biosystems, USA). The reaction conditions were as follows: 95 ºC/10 min, 40 cycles of 95 ºC/15 s, 60 ºC/1 min, and the melting curve: 95 ºC/15 s, 60 ºC/15 s and 95 ºC/15 s. PCR amplification was related to a standard-curve. To the smallest dilution of cDNA standard it was given the relative value 100 and, following the same reason of dilution, the other 3 points were 25, 6.25 and 1.56. The results were expressed as percentage of differences relative to normal controls (relative expression) using 7300 systems SDS software (Applied Biosystems, USA).

2.8. Data analysis

Analyses of variance (ANOVA) and Tukey-Kramer multiple comparison test were used to determine differences between the groups. A probability (*p*) of .05 was chosen as the significant level (Zar, 1999).

3. Results

All melanoma-bearing groups developed a tumor area, reflecting the successful experimental model. Tumors were weighed and no significant differences were seen among the groups due to a high variability. However, stress induced a higher tumor

area, while propolis-treated mice, stressed or not, showed a melanoma development similar to control (data not shown). As to mice behavior, stressed mice (G2) treated with propolis (G4) and bearing melanoma (G6 and G8) were dirty, sweating and with piloerection and tachycardia when leaving the restrainer, in comparison to non-stressed groups. However, a significant increase in corticosterone serum levels (stress indicator) was found only in melanoma-bearing mice submitted to stress (G6) ($F(3.74) = 12.428$, $p<.001$) (Fig. 1).

3.1. Th1 cytokines' expression and production

IL-2 gene expression was significantly inhibited in melanoma-bearing groups, stressed or not (G5 and G6) ($F(2.20) = 26.833$, $p<.05$ and $F(3.02) = 26.833$, $p<.01$, respectively). Melanoma-bearing mice treated with propolis, stressed or not, (G7 and G8) showed a significant increase in IL-2 expression ($F(3.38) = 26.833$, $p<.001$) (Fig. 2), what is associated to the basal production of this cytokine in these groups (Fig. 3).

No significant differences were seen in IL-2 basal production in G1-G4. However, increased levels of IL-2 basal production were found in melanoma-bearing mice (G5-G8) ($F(3.38) = 26.775$, $p<.001$), without differences between stressed mice, treated or not with propolis (Fig. 3).

In Con A-stimulated cultures, stress (G2) did not influence IL-2 production ($p>.05$). On the other hand, propolis-treated mice, stressed or not (G3 and G4), showed a significant inhibition in this cytokine production ($F(3.51) = 21.951$, $p<.001$) (Fig. 3). IL-2 was also inhibited in melanoma-bearing mice (G5) in comparison to control ($F(3.12) = 21.951$, $p<.01$). Stressed animals (G6) showed increased concentrations of

IL-2 in comparison to G5 ($F(3.51) = 21.951$, $p<.001$). Propolis also stimulated IL-2 production by G7 and G8, in comparison to G5, but not significantly (Fig. 3).

mRNA IFN-γ was not detected in the experimental groups. Maybe this cytokine expression occurred under the threshold detection of real-time PCR, or its expression in vivo may have occurred before spleens removal. No differences were found in IFN-γ basal production in G1-G4. Propolis stimulated this cytokine production in melanoma-bearing mice, submitted to stress (G8) ($F(3.38) = 11.838$, $p<.001$). In Con A-stimulated cultures, propolis inhibited IFN-γ production (G3) ($F(3.38) = 75.585$, $p<.001$), as well as in stressed mice (G4) ($F(3.38) = 75.585$, $p<.001$). Melanoma also inhibited IFN-γ production ($F(3.38) = 75.585$, $p<.001$); nevertheless, propolis treatment of melanoma-bearing mice submitted to stress (G8) led to a higher IFN-γ production in comparison to G5 ($F(3.38) = 75.585$, $p<.001$) (Fig. 4).

3.2. Th2 cytokines' expression and production

No alterations were found in IL-4 expression in all groups (Fig. 5). IL-4 basal production was not detected, and no significant differences were seen in this cytokine production in Con A-stimulated cultures (Fig. 6).

Propolis treatment (G3) enhanced significantly IL-10 gene expression (Fig. 7) ($F(3.12) = 16.479$, $p<.01$). Among the melanoma-bearing groups, G5, G6 and G7 showed a significant inhibition in IL-10 expression ($F(2.25) = 16.479$, $p<.05$); however, propolis treatment of melanoma-bearing mice submitted to stress (G8) induced a similar IL-10 expression in comparison to control (G1).

No alterations were seen in IL-10 basal production, and increased basal IL-10 was observed only in G8 ($F(3.38) = 9.972$, $p<.001$) (Fig. 8). In Con A-stimulated

cultures, propolis-treated mice, stressed or not, showed a significant inhibition in IL-10 production ($F(3.38) = 48.577$, $p<.001$). In melanoma-bearing mice stressed or not (G7 and G8), propolis stimulated significantly IL-10 production, ($F(3.38) = 48.577$, $p<.001$) (Fig. 8). Propolis treatment induced higher IL-10 concentrations stimulated or not by Con A in comparison to G5 ($F(3.38) = 48.577$, $p<.001$).

Ethanol (propolis solvent) did not influence cytokines production, and propolis effects were exclusively due to its chemical constituents.

4. Discussion

Propolis has been widely investigated lately, and its effect on stressed mice has been the subject of our group (Sforcin et al., 2008). In this work, Th1 and Th2 cytokines' expression and production were analyzed after B16F10 cells inoculation in mice. Although the importance of stress in life appears to be significant, the cellular and molecular mechanisms involved in the pathophysiology of stress still remain largely unclear, and it is important to investigate how stress can influence Th1/Th2 homeostasis, resulting in resistance or facilitation of tumor progression.

A successful B16F10 cells inoculation was observed in our model, and no metastasis was seen after s.c. tumor inoculation. Sá-Rocha et al. (2006) found metastasis in the lung of mice submitted to social stress after 14 days, but B16F10 cells were inoculated into the tail vein. Stressed mice showed a higher tumor area, whereas propolis treatment did not promote tumor growth.

All groups submitted to stress protocols left the restrainer with tachycardia, piloerection, hyperlocomotion, sweating and dirty. HPA axis has an important role in behavioral and immunological responses during stress, and glucocorticoids play a major regulatory role in long-term stress in mammals. Higher levels of corticosterone were

found only in melanoma-bearing mice submitted to stress. Controversial data on corticosterone concentrations are found in literature, and biological effects depend on the type of stress, intensity, time of measurement of a particular parameter, and mice strains (Kioukia-Fougia et al., 2002). Bowers et al. (2007) related that different stressors commonly used in research may not activate the physiological response to the same extent, and variations in the measured immune responses may reflect differential glucocorticoid activation, differential metabolic pathways, or both processes in response to specific stressors.

Previous observations of our group revealed that stress (immobilization for 7 consecutive days) induced a higher hydrogen peroxide (H_2O_2) generation by peritoneal macrophages. Propolis treatment potentiated H_2O_2 generation and counteracted the alterations found in the spleen of stressed mice (Missima and Sforcin, 2008). However, propolis effect on cytokines' expression and production during chronic stress was little investigated.

Cytokines play an important role in controlling tumor growth and metastasis. A vigorous Th1 response is required for the destruction of tumor cells, while a Th2 response would create a tolerogenic environment in which melanoma could grow (McCarter et al., 2005).

In groups without melanoma, stress did not affect Th1/Th2 cytokines' expression or production, while propolis inhibited Th1 (IFN-γ and IL-2) and Th2 (IL-10) production, suggesting the anti-inflammatory action of this apitherapeutic agent after 14 of administration. Melanoma-bearing mice showed an inhibition in Th1 cytokines production. Thejass and Kuttan (2007) observed decreased IL-2 plasma levels in C57BL/6 mice of B16F10 inoculation. On the other hand, Posevitz et al. (2003) found no consistent changes in the mRNAs of TNF-α, TNF-β, IL-1α, IL-1β, IL-6, IL-4,

IL-5, IFN-γ, IL-2, and IL-3 of C57BL/6 or DBA/2 melanoma-bearing mice submitted to immobilization. Propolis administration to melanoma-bearing mice submitted to stress enhanced IL-2 expression, an increased the production of IFN-γ and IL-2, indicating the activation of antitumor cell-mediated immunity.

IL-2 is known for its potent ability to activate CD8+ T cells and natural killer cells, and several studies have been conducted to investigate the combinations of IL-2 and chemotherapy in advanced melanoma (Fang et al., 2008). IFN-γ might be involved in antitumor and antiangiogenic activities (Corthay et al., 2005). Gollob et al. (2005), through DNA microarray analysis, found that the antimelanoma effect of IFN-γ in human melanoma DM6 cells was associated with the down-regulation of multiple genes involved in G-protein signaling and phospholipase C activation (including Rap2B and calpain 3) as well as the down-regulation of genes involved in melanocyte/melanoma survival (MITF and SLUG), apoptosis inhibition (Bcl2A1 and galectin-3), and cell cycling (CDK2). However, although IFN-γ has been generally thought to enhance antitumor immune responses, it has also been reported that IFN-γ could promote tumor immune evasion (He et al., 2005).

Patients with advancing or progressing melanoma have frequently circulating cytokine levels of Th2 pattern (McCarter et al., 2005). These authors suggested that melanoma does have a direct effect on T cells, but the mechanisms involved in the genesis of a sustained Th2 response in cancer patients is still unclear. IL-10 exerts immunosuppressive effects and may act as a cancer-promoting agent. No changes in IL-4 expression and production were seen. Propolis stimulated IL-10 expression and production, what may be associated to immunoregulatory effects. However, it has been reported that IL-10 also possesses immunostimulatory and anti-angiogenic effects and thus may inhibit tumor growth (Vuoristo, 2007).

In our work, systemic levels of cytokines were not determined, nor melanoma-derived cytokines, but data from our laboratory showed that propolis stimulated pro-inflammatory cytokines production (IL-1β and IL-6) by melanoma-bearing mice submitted to stress (Missima et al., not submitted). TNF-α, IL-1β and IL-6 may act as autocrine growth factors for tumor angiogenesis (Thejass and Kuttan, 2007). However, both tumor-promoting and tumor-suppressing effects of TNF-α have been reported. Corthay et al. (2005) reported that TNF-α may act in synergy with IFN-γ for the activation of tumoricidal macrophages. Since propolis induced higher levels of cytokines in G8, one may speculate that a synergistic effect of IFN-γ and pro-inflammatory cytokines could inhibit tumor growth in vivo by inducing the production of antiangiogenic factors by tumor cells.

Herbal drugs are recognized as cancer chemopreventive agents. Several plant extracts and isolated compounds are well known to suppress tumor cell growth due to apoptosis in different tumor cell lines (Guruvayoorappan and Kuttan, 2007). It has been demonstrated in vitro that propolis possesses antitumoral active compounds, such as caffeic acid phenethyl ester and artepillin C; however, the antiproliferative action of propolis on tumor cells may be the result of the synergistic effect of propolis constituents (Búfalo et al., in press). In vivo, propolis-treated rats showed an increased cytotoxic activity of natural killer cells (Sforcin et al., 2002). Orsolic et al. (2006) suggested that the antitumor activity of propolis and some of its constituents is associated with the augmentation of non-specific antitumor immunity, via macrophages activation, which in turn could produce soluble factors and interfere directly in the tumor cells or in the functions of other immune cells. Propolis administration to rats after treatment with the carcinogen 1,2 dimethylhydrazine reduced significantly the number of aberrant crypt foci, reflecting a suppression of the clonal expansion of the

initiated cells that characterizes the promotion step of carcinogenesis (Bazo et al., 2002).

The polarization of Th1 cells is thought to contribute to cellular immune response and increase CD8+ cytotoxic T lymphocytes, playing an important role in anti-tumor immune surveillance (Hu et al., 2007). Our data indicate that propolis showed an immunomodulatory activity in this assay, what may be related to its antitumoral action in vivo, but this aspect still deserves further investigation to understand propolis efficacy in tumor-bearing mice, mainly during stress.

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Graphs

Fig. 1. Corticosterone concentration (ng/mL). G1: control; G2: stress; G3: propolis; G4: propolis + stress; G5: melanoma; G6: melanoma + stress; G7: melanoma + propolis; G8: melanoma + propolis + stress. Data represent means and standard-deviation of 8 animals. * significantly different from G1 ($p<.001$).

Fig. 2. Relative IL-2 mRNA quantification. Control group received the relative value of 100. G1: control; G2: stress; G3: propolis; G4: propolis + stress; G5: melanoma; G6: melanoma + stress; G7: melanoma + propolis; G8: melanoma + propolis + stress. Data represent means and standard-deviation of 8 animals. * significantly different from G1 ($p<.05$). ** significantly different from G1 ($p<.01$). *** significantly different from G1 ($p<.001$).

Fig. 3. IL-2 production (pg/mL) by spleen cells stimulated or not with concanavalin A (5 µg/mL) for 48 h. G1: control; G2: stress; G3: propolis; G4: propolis + stress; G5: melanoma; G6: melanoma + stress; G7: melanoma + propolis; G8: melanoma + propolis + stress. Data represent means and standard-deviation of 8 animals. * significantly different from G1 ($p<.001$). # significantly different from G1 + Con A

(p<.01). ## significantly different from G1 + Con A (p<.001). ♦ significantly different from G5 + Con A (p<.001).

Fig. 4. IFN-γ production (pg/mL) by spleen cells stimulated or not with concanavalin A (5 μg/mL) for 48 h. G1: control; G2: stress; G3: propolis; G4: propolis + stress; G5: melanoma; G6: melanoma + stress; G7: melanoma + propolis; G8: melanoma + propolis + stress. Data represent means and standard-deviation of 8 animals. * significantly different from G1 (p<.001). # significantly different from G1 + Con A (p<.001).♦ significantly different from G5 + Con A (p<.001).

Fig. 5. Relative IL-4 mRNA quantification. Control group received the relative value of 100. G1: control; G2: stress; G3: propolis; G4: propolis + stress; G5: melanoma; G6: melanoma + stress; G7: melanoma + propolis; G8: melanoma + propolis + stress. Data represent means and standard-deviation of 8 animals.

Fig. 6. IL-4 production (pg/mL) by spleen cells stimulated with concanavalin A (5 μg/mL) for 48 h. G1: control; G2: stress; G3: propolis; G4: propolis + stress; G5: melanoma; G6: melanoma + stress; G7: melanoma + propolis; G8: melanoma + propolis + stress. Data represent means and standard-deviation of 8 animals.

Fig. 7. Relative IL-10 mRNA quantification. Control group received the relative value of 100. G1: control; G2: stress; G3: propolis; G4: propolis + stress; G5: melanoma; G6: melanoma + stress; G7: melanoma + propolis; G8: melanoma + propolis + stress. Data represent means and standard-deviation of 8 animals. * significantly different from G1 (p<.01).

Fig. 8. IL-10 production (pg/mL) by spleen cells stimulated or not with concanavalin A (5 μg/mL) for 48 h. G1: control; G2: stress; G3: propolis; G4: propolis + stress; G5: melanoma; G6: melanoma + stress; G7: melanoma + propolis; G8: melanoma + propolis + stress. Data represent means and standard-deviation of 8 animals. * significantly different from G1 (p<.001).# significantly different from G1 + Con A (p<.001). ♦ significantly different from G5 (p<.001).• significantly different from G5 + Con A (p<.001).

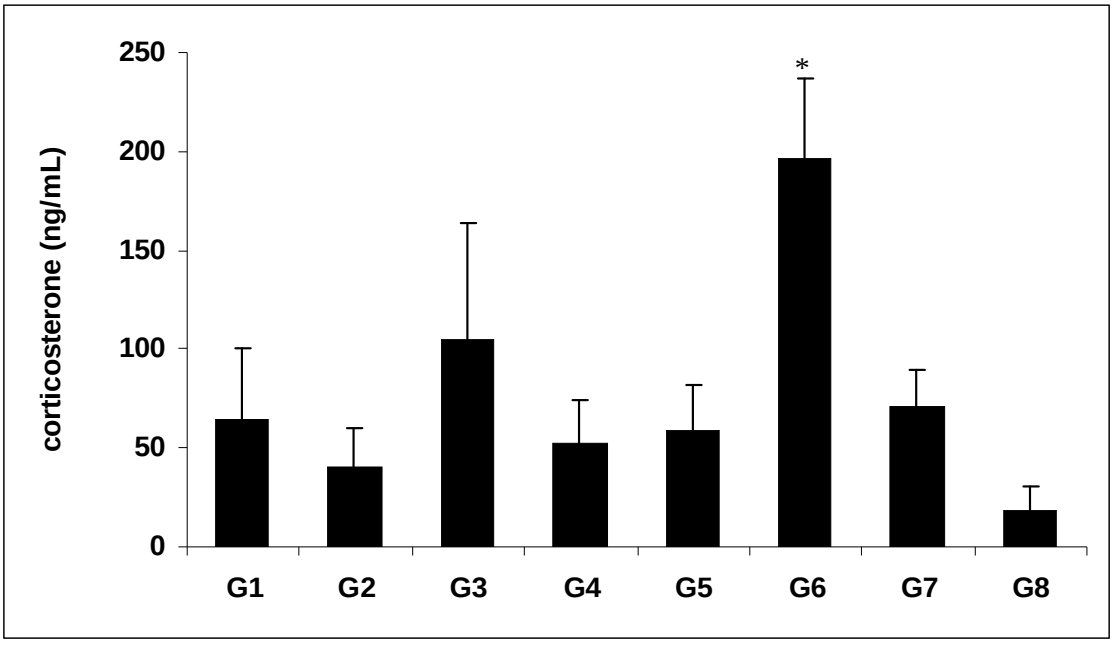
corticosterone (ng/mL)
250
200
150
100
50
0
*
G1
G2
G3
G4
G5
G6
G7
G8

Fig. 1.

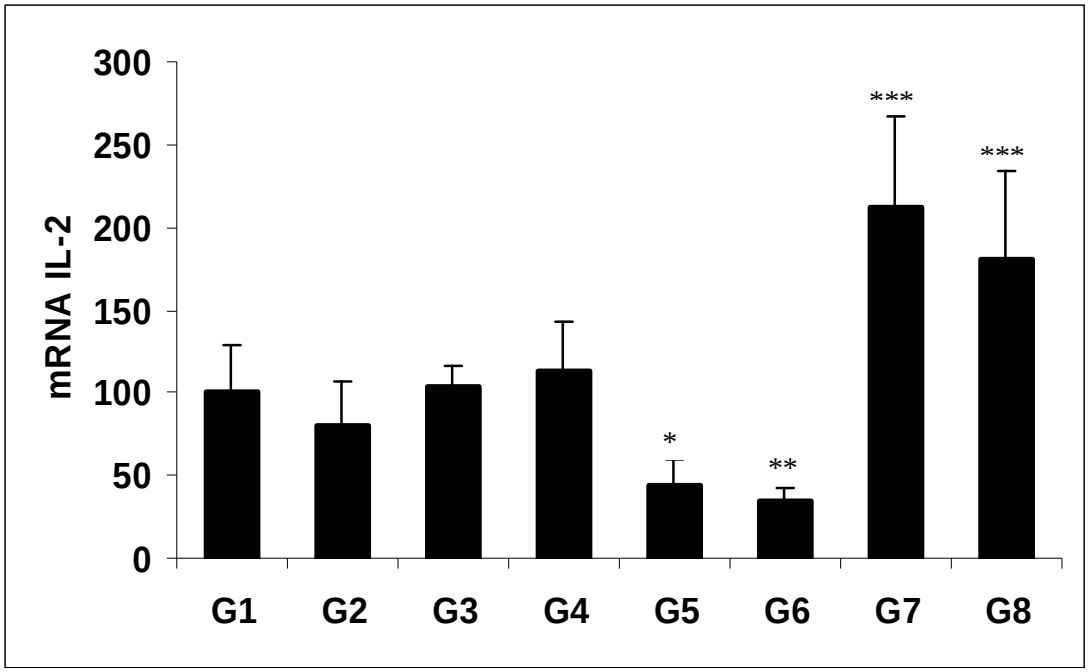
mRNA IL-2
300
250
200
150
100
50
0
*
**
***
***
G1
G2
G3
G4
G5
G6
G7
G8

Fig. 2.

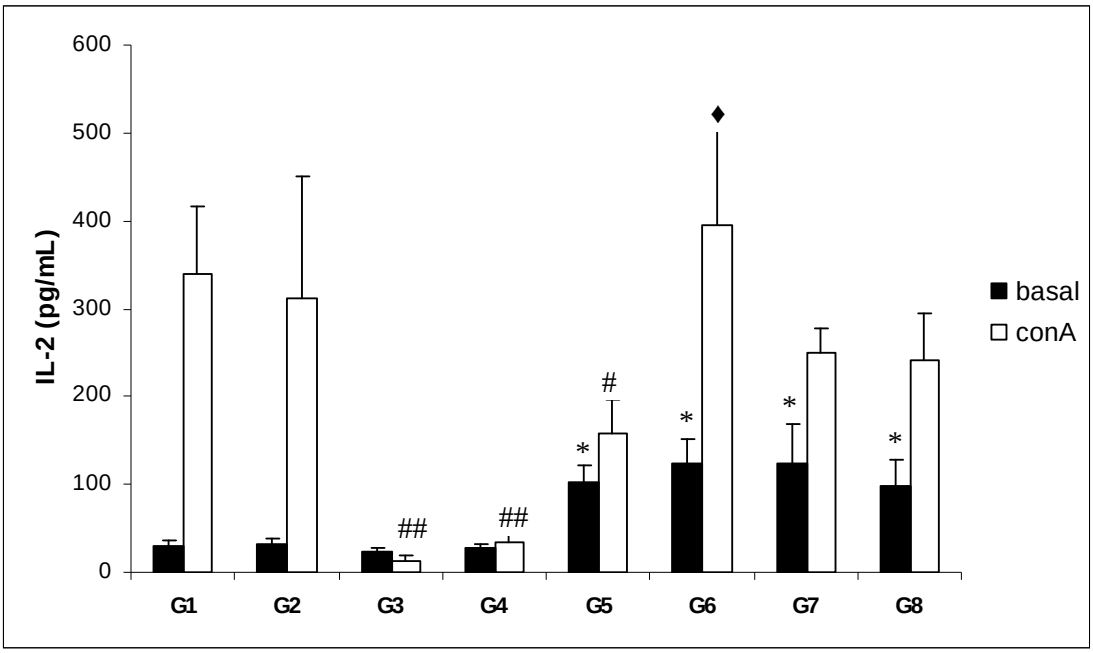
600
500
400
300
200
100
0
IL-2 (pg/mL)
basal
conA
##
##
*
#
*
♦
*
*
G1
G2
G3
G4
G5
G6
G7
G8

Fig. 3.

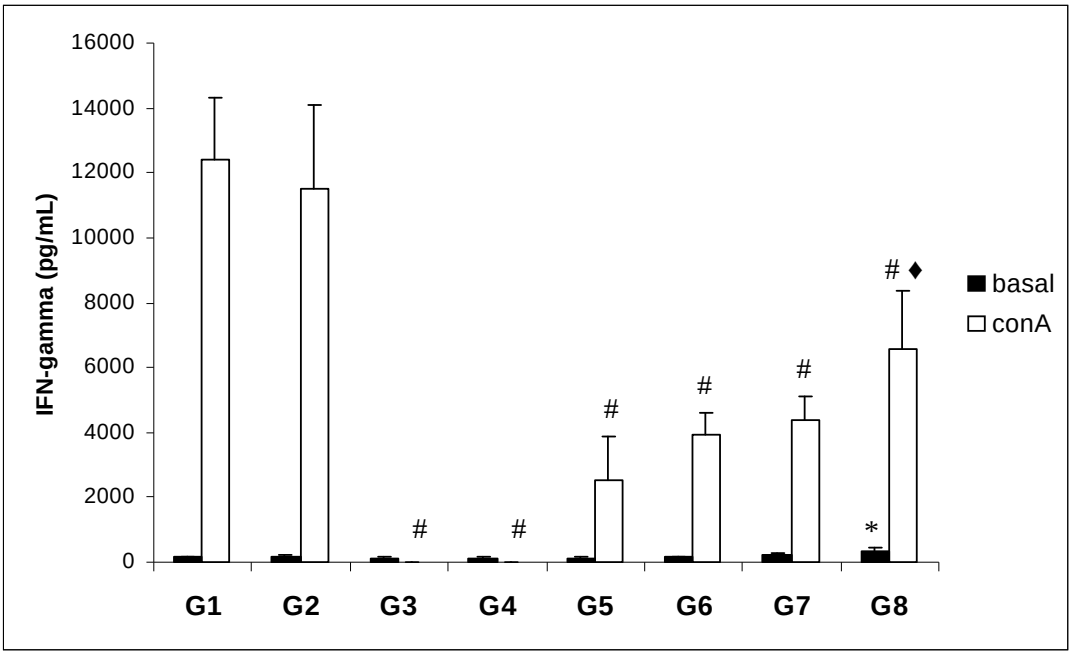
16000
14000
12000
10000
8000
6000
4000
2000
0
IFN-gamma (pg/mL)
basal
conA
#
#
#
#
#
*
♦
G1
G2
G3
G4
G5
G6
G7
G8

Fig. 4.

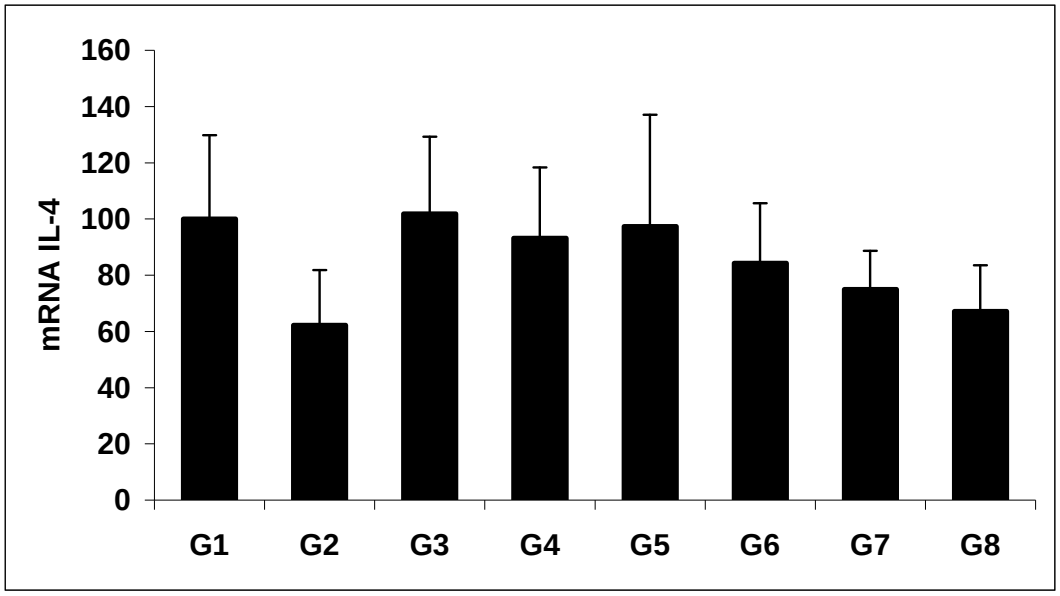
160
140
120
100
80
60
40
20
0
mRNA IL-4
G1
G2
G3
G4
G5
G6
G7
G8

Fig. 5.

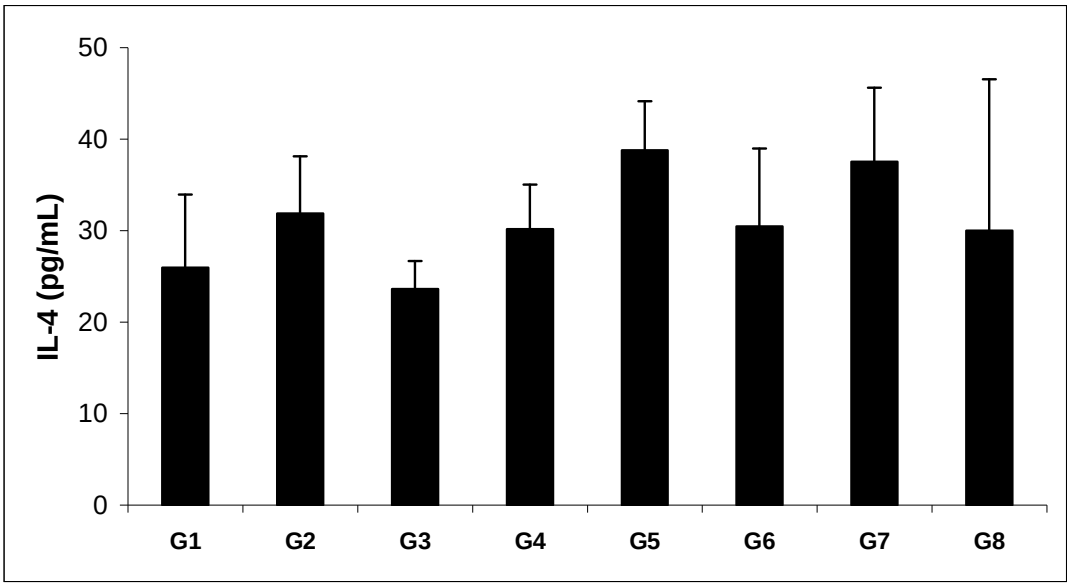
50
40
30
20
10
0
IL-4 (pg/mL)
G1
G2
G3
G4
G5
G6
G7
G8

Fig.6.

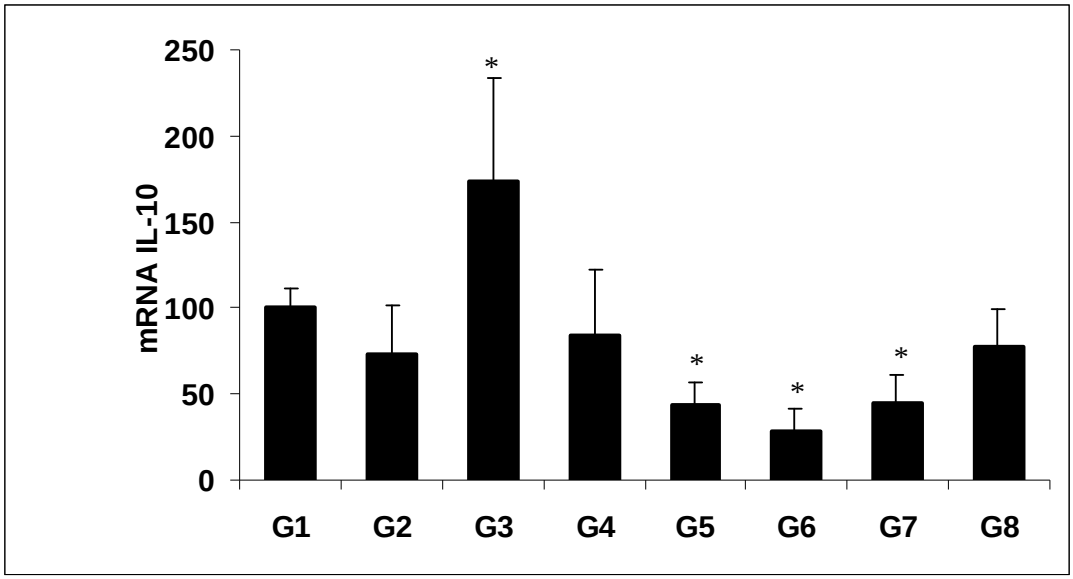
mRNA IL-10
250
200
150
100
50
0
*
*
*
*
G1
G2
G3
G4
G5
G6
G7
G8

Fig. 7.

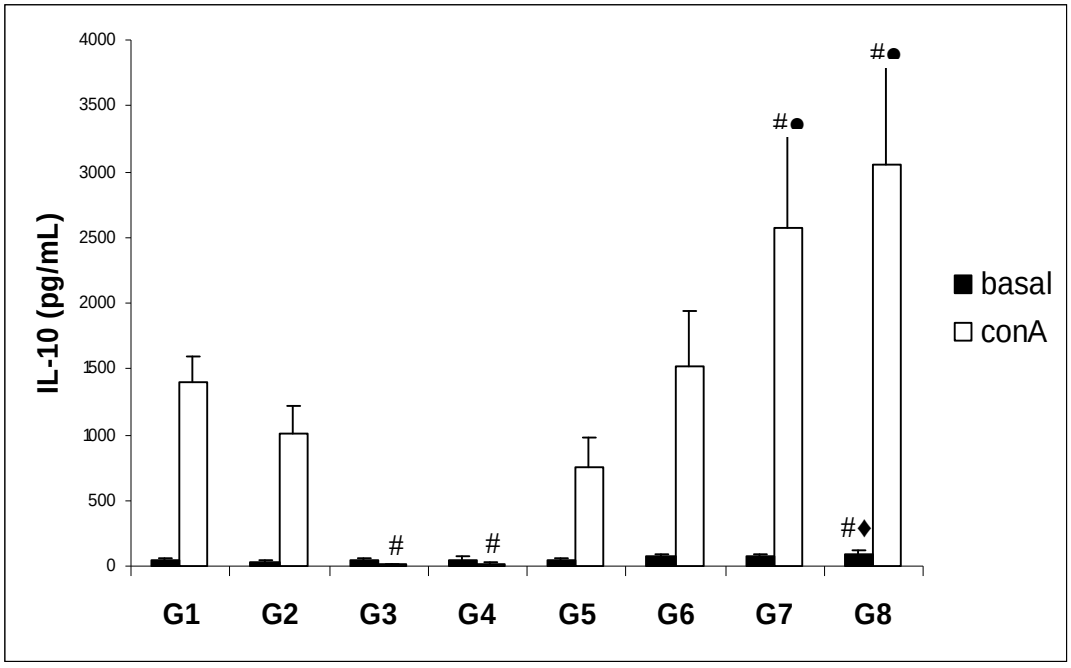
IL-10 (pg/mL)
4000
3500
3000
2500
2000
1500
1000
500
0
#
#
#●
#●
#♦
basal
conA
G1
G2
G3
G4
G5
G6
G7
G8

Fig. 8.

*Manuscrito 2**

De acordo com as normas da revista Journal of ApiProduct and ApiMedical Science. As normas constam no anexo 2 deste trabalho (página 72).

Propolis effect on pro-inflammatory cytokines produced by melanoma-bearing mice submitted to chronic stress

Running title: Propolis effect on melanoma-bearing mice

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Summary

Altered levels of pro-inflammatory cytokines could be prometastatic and proangiogenic factors. Stress leads to somatic disorders such as cancer, and animal models have been used to investigate stress effects on tumorigenesis and immune response modulation. Propolis is a honeybee product and its immunomodulatory effects on stressed mice have been the goal of our recent researches. In this work, propolis effect on pro-inflammatory (IL-1β and IL-6) cytokines production by melanoma-bearing mice submitted to immobilization stress were analyzed. Propolis administration to stressed mice inhibited pro-inflammatory cytokines production. On the other hand, propolis induced higher levels of IL-1β and IL-6 in melanoma-bearing mice submitted or not to chronic stress. Since propolis also stimulated Th1 cytokines production in these mice, one may speculate that a synergistic effect of IFN-γ and pro-inflammatory cytokines could inhibit tumor growth in vivo by inducing the production of antiangiogenic factors. Further investigation will help to understand propolis usefulness during stress.

Keywords: Interleukin-1β; Interleukin-6; Stress; Melanoma; Propolis; Immunomodulation

Introduction

Propolis is a resinous hive product, used in folk medicine since ancient times, due to its many biological properties, such as antimicrobial (Sforcin *et al.*, 2000; Freitas *et al.*, 2006; Búfalo *et al.*, submitted), antitumor (Banskota *et al.*, 2001; Bazo *et al.*, 2002), immunomodulatory (Sforcin, 2007), among others. Analyses of its chemical composition have identified at least 300 compounds in propolis (De Castro, 2001), and its effects on stressed mice have been investigated by our research group (Missima and Sforcin, 2008).

Stress comprises several nonspecific events, altering the homeostatic state of the organism and leading to "sickness behavior". Stress responses induce co-activation of both sympathoadrenomedullary system and hypothalamic-pituitary-adrenal (HPA) axis, with consequent release of catecholamines and glucocorticoids, respectively (Dhabhar, 2002). Psychological and behavioral changes are associated to physiological changes, evidencing a communication between the immune, endocrine and central nervous systems during stress.

Catecholamines, glucocorticoids and pro-inflammatory cytokines are considered among the principal messengers responsible for the bi-directional communication between the central nervous system and the immune system (Maes *et al.*, 1998). IL-1β, also called endogenous pyrogen, is synthesized primarily by monocytes and macrophages, and contributes to the pathogenesis of chronic inflammatory diseases (Pope and Tschopp, 2007). IL-6 is a typical pleiotropic cytokine, playing an important role in the homeostasis of the immune system. IL-6 is one of the major cytokines that stimulates the HPA axis during inflammatory stress (Sjogren *et al.*, 2006).

Because of glucocorticoids' immunosuppressive effects, stress plays a role in the etiology of many diseases, such as cancer, being detrimental to health. Melanoma is among the most immunogenic of all solid cancers, and the presence of tumor antigen-specific antibodies and tumor-specific cytotoxic T cells in the peripheral blood of melanoma patients has been well established (Fang *et al.*, 2008). B16F10 is a selective variant cell line obtained from pulmonary metastasis of a melanoma, syngeneic to black C57BL/6 mice (Sá-Rocha *et al.*, 2006). Moreover, altered levels of IL-1β, IL-6 and TNF-α could be prometastatic and proangiogenic factors and their deregulated expression directly correlates with the metastatic potential of several forms of cancer.

Whereas the relationship between stress and immunity has been extensively studied in the last decades, the use of natural products during stress deserves investigation, mainly in melanoma-bearing mice. We report herein propolis' immunomodulatory action on pro-inflammatory (IL-1β and IL-6) cytokines production by melanoma-bearing mice submitted to chronic stress.

Materials and Methods

Propolis sample

Propolis was collected in the Beekeeping Section, UNESP. Propolis was ground and 30% ethanolic extracts of propolis were prepared (30 g of propolis, completing the volume to 100 mL with 70% ethanol), in the absence of bright light, at room temperature, with moderate shaking. After a week, extracts were filtered and the dry weight of the extracts was calculated (120 mg/mL) (Sforcin *et al.*, 2005). Propolis chemical composition was investigated using thin-layer chromatography (TLC), gas-

chromatography (GC), and gas chromatography-mass spectrometry (GC-MS) analysis (Bankova *et al.*, 1998).

Melanoma cells

B16F10 cells were cultured in DMEM (Cultilab, Campinas, SP, Brazil), supplemented with 25 mM HEPES (Sigma – Aldrich, St. Louis, MO, USA) and 10% fetal calf serum. Cell suspensions were detached from the culture flasks using 0.2% trypsin, and viable cells were counted using a hemocytometer. Mice were inoculated with 5 x 10^4 cells in 0.1 mL of phosphate-buffered saline subcutaneously (s.c.) into the right flank region. Tumor development was monitored weekly.

Animals, experimental groups and stress procedure

C57BL/6 male mice aged between 8 and 12 weeks were kept in rooms at 21-25ºC, with a 12 h /12 h light/dark cycle. Food and water were provided *ad libitum*. This work agrees with Ethical Principles in Animal Research adopted by Brazilian College of Animal Experimentation (nº 464).

Mice were divided into 8 groups (G1, G2, G3, G4, G5, G6, G7, G8), of 8 animals each. G1 was considered as control, and received physiologic solution (NaCl 0.9%, 0.1 mL). G2 was submitted to restraint stress in a well-ventilated immobilization tube (restrainer) of about 50 mL capacity for 15, 30, 45, 60, 75, 90 and 120 minutes during 7 consecutive days, and from 7^{th} to 14^{th} day, mice were submitted to restraint stress for 2 hours a day, at a fixed time between 8.00 and 11.00 a.m. This procedure is easily performed and causes no physical pain to the animals (Dominguez-Gerpe and Rey-Méndez, 2001; Sforcin *et al.*, 2007).

G3 was treated daily with propolis (200 mg/kg in 0.1 mL, orally), and G4 was treated daily with propolis and submitted to the same stress protocol (Missima and Sforcin, 2008). After 24 h of the respective treatments, animals were sacrificed using a CO_2 inhalation chamber.

G5 was inoculated with B16F10 (5×10^4 cells) s.c. into the right flank. G6 was inoculated with B16F10 cells and submitted to stress. G7 was inoculated with B16F10 cells and treated daily with propolis. G8 was inoculated with B16F10 cells, treated daily with propolis and submitted to stress. All groups had no water and food during stress. After 14 days of melanoma inoculation, mice were sacrificed and metastases were investigated.

70% ethanol (propolis solvent) effects were also investigated after its administration for 14 days to mice.

Corticosterone determination

Before sacrifice, blood was collected by cardiac puncture and serum was stored at –20°C. Corticosterone concentrations were determined by radioimmunoassay, using a commercial kit (Coat-A-count, DPC, Los Angeles, CA, USA).

Spleen cells cultures and cytokines determination

After sacrifice, spleens were aseptically removed and cells were suspended at a concentration of 5×10^6/mL in RPMI 1640 (Cultilab, Campinas, SP, Brazil) supplemented with 10% fetal calf serum and 1% L-glutamine and cultured in flat-bottomed 24-well plates. Cells were cultured in triplicates (1 mL/well) and stimulated with lipopolysaccharide (LPS – 5 μg/mL) for 48h at 37ºC and 5% CO_2.

Supernatants of spleen cell cultures were collected and assayed for IL-1β and IL-6 cytokines determination by enzyme-linked immunosorbent assay (ELISA), according to manufacturer's instructions (BD Biosciences, San Diego, USA). Briefly, a 96-well flat bottom Maxisorp (Nunc, USA) was coated with capture antibody specific to each cytokine. The plate was washed and blocked before 100 µL of the supernatants and serially diluted specific standards were added to the respective wells. Following a series of washing, the captured cytokine was detected using the specific conjugated detection antibody. The substrate reagent was added into each well and, after color development, the plate was read at 450 nm, using an ELISA plate reader (Tan *et al.*, 2006).

Statistical analysis

Analyses of variance (ANOVA) and Tukey-Kramer multiple comparison test were used to determine differences between the groups. A probability (p) of 0.05 was chosen as the significant level (Zar, 1999).

Results

All melanoma-bearing groups developed a tumor area, reflecting a successful experimental model. Tumors were weighed and no significant differences were seen among the groups due to a high variability. However, stress induced a higher tumor area, while propolis-treated mice, stressed or not, showed a melanoma development similar to control (data not shown). As to mice behavior, stressed mice (G2) treated with propolis (G4) and bearing melanoma (G6 and G8) were dirty, sweating and with piloerection and tachycardia when leaving the restrainer, in comparison to non-stressed

groups. However, a significant increase in corticosterone serum levels (stress indicator) was found only in melanoma-bearing mice submitted to stress ($p<0.001$) (Fig. 1).

IL-1β concentration was detected only in LPS-stimulated cultures. Propolis-treated mice and stressed or not (G3 and G4) showed a significant inhibition this cytokine production ($p<0.01$). In melanoma-bearing groups, increasing concentrations of IL-1β were seen in propolis-treated mice submitted or not to stress (G7 and G8) (Fig. 2).

IL-6 basal production was elevated only melanoma-bearing groups ($p<0.05$). In LPS-stimulated cultures, propolis treatment of stressed mice (G4) inhibited IL-6 production ($p<0.05$). On the other hand, increased concentrations of IL-6 ($p<0.05$) were found after propolis administration to melanoma-bearing mice submitted or not to stress (G7 and G8) (Fig. 3).

Ethanol (propolis solvent) did not influence cytokines nor corticosterone production, and propolis effects were exclusively due to its chemical constituents.

Discussion

Propolis action on stressed mice has attracted our group's interest (Missima and Sforcin, 2008; Sforcin *et al.,* 2008). Since propolis effect on cytokines production during stress was little investigated, pro-inflammatory cytokines production was analyzed after B16F10 cells inoculation in stressed mice.

A successful B16F10 cells inoculation was observed in our model, and no metastasis was seen after subcutaneous tumor inoculation. Although no significant differences were seen in tumor weight among the groups, stress induced a higher tumor

area, whereas propolis treatment did not affect tumor growth. Metastasis was found in the lung of mice submitted to social stress after 14 days when B16F10 cells were inoculated into the tail vein (Sá-Rocha *et al*., 2006).

Pro-inflammatory cytokines are known to activate the HPA axis and consequently increase glucocorticoids levels. However, stressed mice (G2) showed no alterations in IL-1β, IL-6 and corticosterone concentrations. With respect to mice behavior, stressed groups (G2, G4, G6 and G8) were dirty, sweating and with piloerection when leaving the restrainer, but higher levels of corticosterone were found only in melanoma-bearing mice submitted to stress (G6), what was associated to increased IL-6 production. HPA axis has an important role in behavioral and immunological responses during stress, but controversial data on corticosterone concentrations are found in literature. Moreover, biological effects depend on the intensity and type of stress, time of measurement of a particular parameter, and mice strains (Kioukia-Fougia *et al.*, 2002). Different stressors may not activate the physiological response to the same extent, and variations in immunological parameters reflect differential glucocorticoid activation and metabolic pathways in response to specific stressors (Bowers *et al.*, 2008).

Pro-inflammatory cytokines have overlapping activities, and their production is also increased in acute inflammatory responses associated with infection, injury, trauma and stress (Kamimura *et al.*, 2003; Avitsur *et al.*, 2006). Pro-inflammatory cytokines could be also prometastatic and proangiogenic factors (Thejass and Kuttan, 2007). Cytokines play an important role in controlling tumor growth and metastasis. A vigorous Th1 response is required for the destruction of tumor cells, while a Th2 response would create a tolerogenic environment in which melanoma could grow (McCarter *et al.*, 2005). Works of our laboratory revealed that propolis stimulated IFN-γ

and IL-2 production by melanoma-bearing mice submitted to stress (G8), indicating the activation of antitumor cell-mediated immunity (Missima *et al.*, not submitted). In this work, propolis administration to G8 induced higher levels of IL-1β and IL-6. Both tumor-promoting and tumor-suppressing effects of TNF-α have been reported. TNF-α may act in synergy with IFN-γ for the activation of tumoricidal macrophages (Corthay *et al.*, 2005). Since propolis stimulated pro-inflammatory and Th1 cytokines production in G8, one may speculate that a synergistic effect of IFN-γ and pro-inflammatory cytokines could inhibit tumor growth in vivo by inducing the production of antiangiogenic factors. Our results are in agreement with other authors, who related that changes in the production of the pro-inflammatory cytokines and IFN-γ take part in the homeostatic responses to psychosocial stress in humans, and that stress-induced anxiety is related to a Th1 response (Maes *et al.*, 1998).

Several researchers have reported the antitumoral property of propolis in vivo and in vitro. Propolis antiproliferative activity on tumor cells has been demonstrated and some responsible compounds were isolated (Banskota *et al.*, 2001; Sforcin *et al.*, 2002; Orsolic *et al.*, 2006; Sforcin, 2007). On the basis of these findings, our data suggest that propolis exerted a possible antitumoral action in vivo enhancing pro-inflammatory and IFN-γ production, but further investigation is still needed to evaluate propolis usefulness in tumor-bearing mice, during stress conditions.

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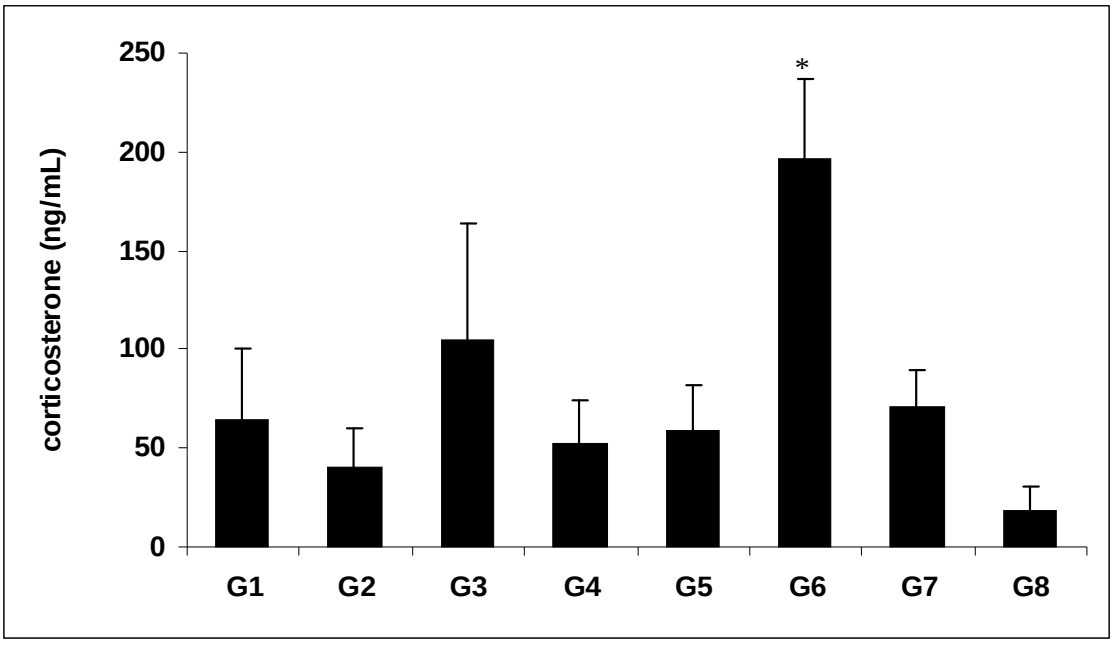
Graphs

Fig. 1. Corticosterone concentrations (ng/mL). G1: control; G2: stress; G3: propolis; G4: propolis + stress; G5: melanoma; G6: melanoma + stress; G7: melanoma + propolis; G8: melanoma + propolis + stress. Data represent means and standard-deviation of 8 animals. * significantly different from G1 ($p<0.001$).

Fig. 2. IL-1β production (pg/mL) by spleen cells stimulated with LPS (5 μg/mL) for 48 h. G1: control; G2: stress; G3: propolis; G4: propolis + stress; G5: melanoma; G6: melanoma + stress; G7: melanoma + propolis; G8: melanoma + propolis + stress. Data represent means and standard-deviation of 8 animals. * significantly different from G1 ($p<0.01$).

Fig. 3. IL-6 production (pg/mL) by spleen cells stimulated or not with LPS (5 μg/mL) for 48 h. G1: control; G2: stress; G3: propolis; G4: propolis + stress; G5: melanoma; G6: melanoma + stress; G7: melanoma + propolis; G8: melanoma + propolis + stress. Data represent means and standard-deviation of 8 animals. * significantly different from G1 ($p<0.05$); $^{\#}$ significantly different from G1 + LPS ($p<0.05$).



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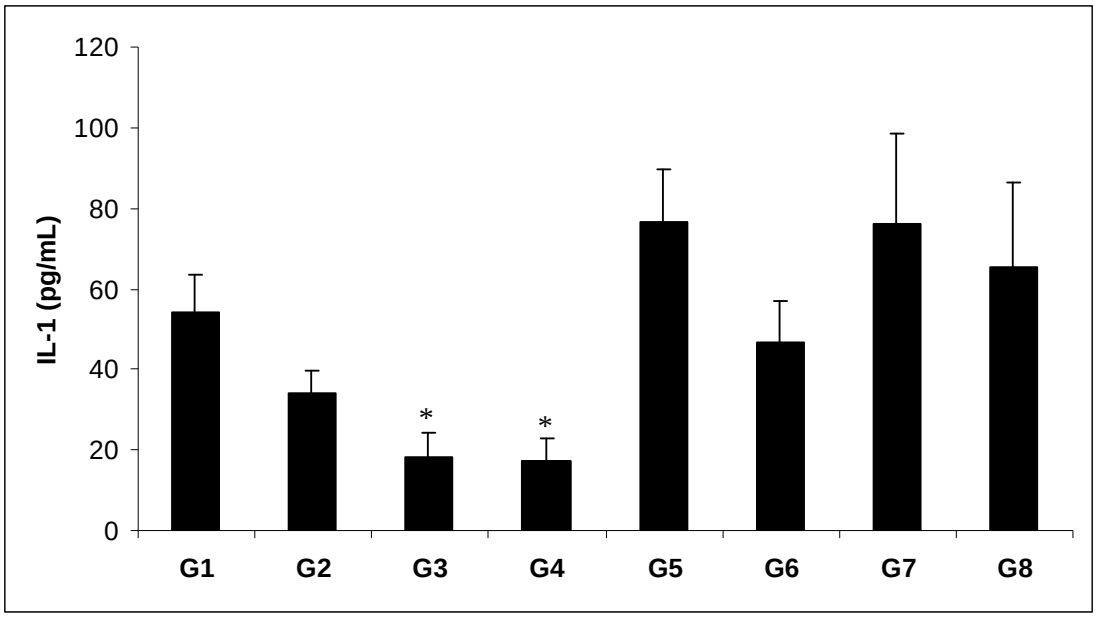

Fig. 2.

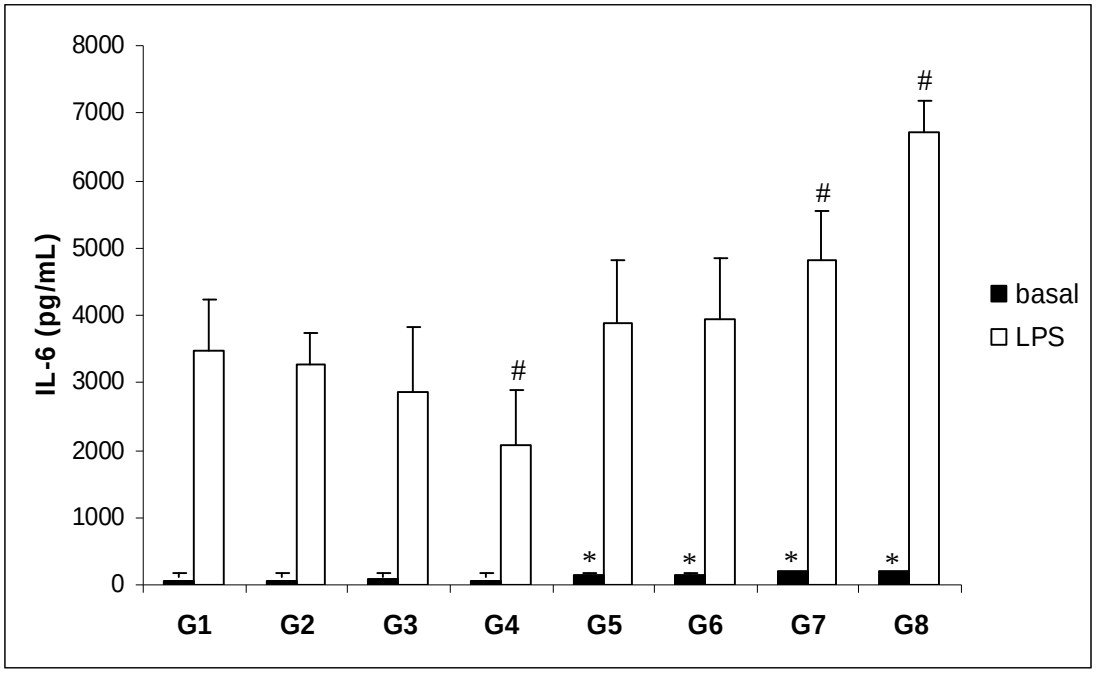
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Fig. 3.

Anexo 1

BRAIN, BEHAVIOR, AND IMMUNITY

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Anexo 2

Journal of ApiProduct and ApiMedical Science

JAAS

The international link between bee product science and practice

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