Journal of ApiProduct and ApiMedical Science 1 (1): 11-15 (2009)
DOI 10.3896/IBRA.4.01.1.05

ORIGINAL RESEARCH ARTICLE

The effect of propolis on pro-inflammatory cytokines produced by melanoma-bearing mice submitted to chronic stress.

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Received 18 December 2008, accepted subject to revision 19 January 2009, accepted for publication 21 January 2009.

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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, the effects of propolis on pro-inflammatory (IL-1β and IL-6) cytokines production by melanoma-bearing mice submitted to immobilization stress were analysed. 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 tumour 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, UNESP, Brazil, *pers. comm.*), antitumour (Banskota *et al.*, 2001; Bazo *et al.*, 2002), immunomodulatory effects (Sforcin, 2007), among others. Analysis 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 behaviour". 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 tumour antigen-specific antibodies and tumour-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 about the immunomodulatory action of propolis 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% foetal calf serum. Cell suspensions were detached from the culture flasks using 0.2% trypsin, and viable cells were counted using a haemocytometer. 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. Tumour 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 agreed 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), previously standardized in our laboratory (Missima and Sforcin, 2008). G4 was treated daily with propolis and submitted to the same stress protocol. After 24 h of the respective treatments, animals were sacrificed using a CO_2 inhalation chamber.

G5 was inoculated with B16F10 (5 x 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 –20ºC. 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 x 10^6/mL in RPMI 1640 (Cultilab, Campinas, SP, Brazil) supplemented with 10% foetal 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 colour 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

The main constituents of our propolis sample, investigated by TLC, GC and GC-MS analysis, were: flavonoids (kaempferid, 5,6,7-trihydroxy-3,4'-dimethoxyflavone, aromadendrine-4'-methyl ether); a prenylated *p*-coumaric acid and two benzopyranes: *E* and *Z* 2,2-dimethyl-6-carboxyethenyl-8-prenyl-2H-benzopyranes); essential oils (spathulenol, (2Z,6E)-farnesol, benzyl benzoate and prenylated acetophenones); aromatic acids (dihydrocinnamic acid, p-coumaric acid, ferulic acid, caffeic acid, 3,5-diprenyl-p-coumaric acid, 2,2-dimethyl-6-carboxy-ethenyl-8-prenyl-2H-1-benzo-pyran); di- and triterpenes, among others (Bankova *et al.*, 1998). The main vegetal source of propolis samples in Botucatu, SP, Brazil, is *Baccharis dracunculifolia* DC., followed by *Eucalyptus citriodora* Hook and *Araucaria angustifolia* (Bert.) O. Kuntze (Bankova *et al.,* 1999).

All melanoma-bearing groups developed a tumour area, reflecting a successful experimental model, and no metastasis was seen after 14 days of B16F10 cells inoculation. Tumours were weighed and no significant differences were seen among the groups due to a high variability. However, stress induced a higher tumour 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.

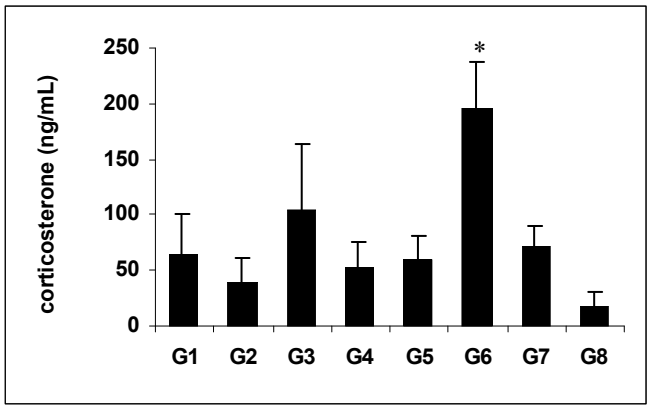

Fig. 1. Corticosterone concentrations (ng/mL). G1: control; G2: stress; G3: propolis; G4: propolis + stress; G5: melanoma; G6: melanoma + stress; G7: melanoma + propolis; G8: melanoma + propolis + stress. Data represent means and standard-deviation of 8 animals. * significantly different from G1 ($p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

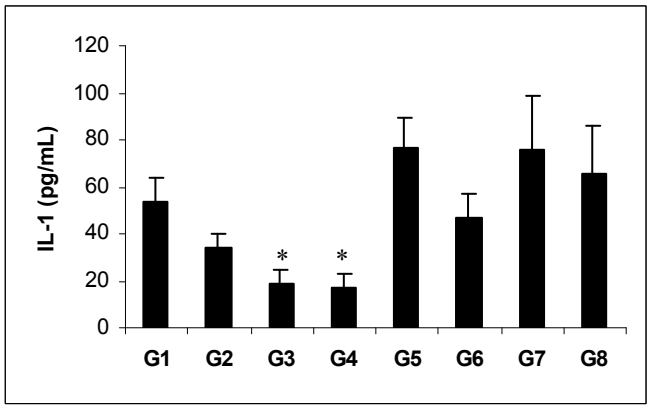

Fig. 2. IL-1β production (pg/mL) by spleen cells stimulated with LPS (5 µg/mL) for 48 h. G1: control; G2: stress; G3: propolis; G4: propolis + stress; G5: melanoma; G6: melanoma + stress; G7: melanoma + propolis; G8: melanoma + propolis + stress. Data represent means and standard-deviation of 8 animals.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

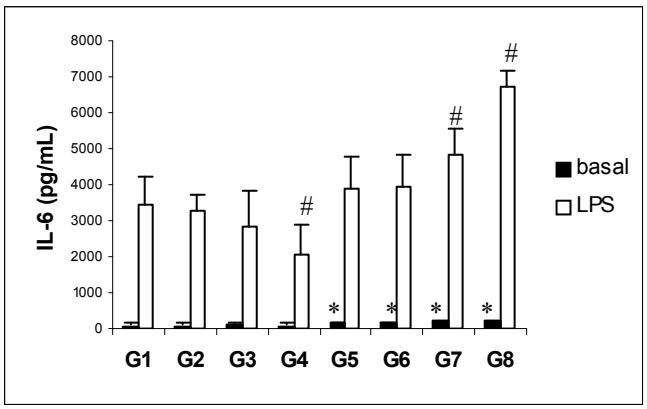

Fig. 3. IL-6 production (pg/mL) by spleen cells stimulated or not with LPS (5 µg/mL) for 48 h. G1: control; G2: stress; G3: propolis; G4: propolis + stress; G5: melanoma; G6: melanoma + stress; G7: melanoma + propolis; G8: melanoma + propolis + stress. Data represent means and standard-deviation of 8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

Discussion

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

A successful B16F10 cells inoculation was observed in our model, and no metastasis was seen after subcutaneous tumour inoculation. Although no significant differences were seen in tumour weight among the groups, stress induced a higher tumour area, whereas propolis treatment did not affect tumour 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 behaviour, 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 tumour growth and metastasis. A vigorous Th1 response is required for the destruction of tumour cells, while a Th2 response would create a tolerogenic environment in which melanoma could grow (McCarter *et al.*, 2005). Data from our laboratory revealed that propolis stimulated IFN-γ and IL-2 production by melanoma-bearing mice submitted to stress (G8), indicating the activation of antitumour cell-mediated immunity (Missima, UNESP, Brazil, *pers. comm.*). In this study, propolis administration to G8 induced higher levels of IL-1β and IL-6. Both tumour-promoting and tumour-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 tumour 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 tumour 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 tumoricidal action in *vivo* enhancing pro-inflammatory and IFN-γ production, but further investigation is still needed to evaluate propolis usefulness in tumour-bearing mice, during stress conditions.

Acknowledgements

This work was supported by FAPESP (2005/52566-1) and CAPES (199-01/2007). Authors wish to thank Dr. Lídia Raquel de Carvalho, IB, UNESP, Campus of Botucatu, for the statistical analysis.

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