Effect of the ethanolic extract from green propolis on production of antibodies after immunization against canine parvovirus (CPV) and canine coronavirus (CCoV)

Efeito do extrato etanólico de própolis verde sobre a produção de anticorpos após imunização contra parvovírus canino (CPV) e coronavírus canino (CCoV)

Lílian das Neves FERREIRA[1]; Paula Fonseca FINGER[1]; Clarissa Caetano de CASTRO[1]; Bianca Sica SIEDLER[1]; Lívia Silveira MUNHOZ[1]; Gilberto D'Avila VARGAS[1]; Geferson FISCHER[1]; Silvia de Oliveira HÜBNER[1]

[1]Departamento de Veterinária Preventiva da Faculdade de Medicina Veterinária da Universidade Federal de Pelotas, Pelotas-RS, Brasil

Abstract

This study was designed to evaluate whether an ethanolic extract of green propolis (EEP) can interfere with production of specific antibodies after immunization against parvovirus (CPV) and canine coronavirus (CCoV). Mice were vaccinated with CPV and CCoV (0.75, 1.5 and 3 x 10^6 $TCID_{50}$) with or without 400 μg/dose of the EEP. Twenty one days after the third dose was measured serum IgG. The co-administration of the EEP significantly enhanced serum specific IgG responses to CPV in animals inoculated with the highest concentration of the antigen, and had no influence on levels of antibodies to CCoV. The results indicate that the EEP has immunomodulatory action closely dependent on the type and concentration of antigen used, being able to increase the levels of antibodies to CPV.

Keywords: Propolis. Th1/Th2. Immunomodulation. Vaccine. Canine parvovirus. Canine coronavirus.

Resumo

Este estudo foi realizado para avaliar se extrato etanólico de própolis verde (EEP) pode interferir na produção de anticorpos específicos após imunização contra parvovírus (CPV) e coronavírus canino (CCoV). Camundongos foram vacinados com CPV e CCoV (0.75, 1.5 e 3 x 10^6 $TCID_{50}$) com ou sem 400 μg/dose de EEP. Vinte e um dias após a terceira dose foi mensurado IgG sérica. A coadministração de EEP aumentou significativamente os níveis de IgG específica para o CPV em animais inoculados com a maior concentração do antígeno, e não teve influência sobre os níveis de anticorpos para CCoV. Os resultados indicam que o EEP tem ação imunomoduladora intimamente dependente do tipo e concentração do antígeno utilizado, sendo capaz de aumentar os níveis de anticorpos contra CPV.

Palavras-chave: Própolis. Th1/Th2. Imunomodulação. Vacina. Parvovírus canino. Coronavírus canino.

Introduction

Propolis is a natural substance produced by honey bees from different parts of plants[1,2]. Nowadays there are increasing scientific studies in view of its high pharmacological potential and immunomodulator effects[3,4,5,6,7]. The chemical composition is complex and depends on the flora in the areas where it is collected[2,8]. However, researches indicate that phenolics compounds are the components with remarkable biological activity[9]. The green propolis, produced from a plant of Brazil commonly known as "Alecrim do Campo" (*Baccharis dracunculifolia,*) shows high levels of phenolic compounds such as artepillin C, in addition to cinnamic acid and flavonoids such as pinobanksin and kaempferol[10,11,12].

Since the efficacy of certain vaccines, especially those containing multiple antigens, has been some-

Correspondence to:
Silvia de Oliveira Hübner
Faculdade de Veterinária, Departamento de Veterinária Preventiva
Universidade Federal de Pelotas
Campus Capão do Leão, Caixa Postal 354
CEP: 96010-900 – Pelotas - RS
e-mail: sohubner@yahoo.com.br

Received: 05/04/2011
Approved: 18/04/2012

times questioned[13], the administration associated with an adjuvant or an appropriate immunomodulator can mean a better performance of these vaccines[14]. Thus, the development of vaccines will be highly benefited with the identification of new substances capable of promoting and directing to a proper immune response. This study was designed to evaluate whether an ethanolic extract of green propolis (EEP) rich in phenolic compounds can interfere with production of serum specific antibodies from mice inoculated with different concentrations of canine parvovirus (CPV) and canine coronavirus (CCoV).

Material and Method

Green propolis was obtained from Nectar Farmaceutica Ltda. (Brazil) and stored at –20 ºC. The ethanolic extract was prepared as previously described[15]. After evaporation of the solvent the resulting dried matter was dissolved in phosphate buffer solution (pH 6.2), in a final concentration of 40mg/ml. The chemical composition of the green propolis extract was determined by high performance liquid chromatography (HPLC), as described[7]. The HPLC analysis showed high levels of the phenolic compounds 3,5-diprenyl-4-hydroxycinnamic acid (artepillinC), 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopiran, 3-prenyl-4-hydroxycinnamic acid, p-coumaric acid, caffeic acid, ferulic acid, besides cinnamic acid and the flavonoids pinobanksin and kaempferol. In this sample of green propolis, the flavonoids corresponded to 22.37% of the dried extract.

In this study Swiss mice (*Mus musculus)* female, 4-6 week-old supplied by Bioterio Central of UFPel (Pelotas – Brazil) were used. The animals remained isolated, in controlled environment with temperature between 22-24°C, receiving feeding and water ad libitum. The experiment was approved by the UFPel Committee of Ethics in Animal Experimentation. The euthanasia was performed according to the norms established by resolution number 714 of 20 June 2002, by Conselho Federal de Medicina Veterinaria[16].

The EEP was associated with a commercial canine combination vaccine containing CPV modified-live and inactivated CCoV and adjuvanted with $Al(OH)_3$, in addition to other attenuated virus (canine adenovirus, canine distemper virus and canine parainfluenza virus).

The mice were allocated into eight groups of 10 animals and inoculated with the vaccine subcutaneously at days 0, 30 and 60. Groups one and two received phosphate buffered saline solution (PBS, pH 7.2) or PBS with EEP (400 µg/dose), respectively (negative controls). Group three, four and five received vaccine with $0.75x10^6$, $1.5x10^6$ e $3x10^6$ $TCID_{50}$ (tissue culture infections dose 50%/25 µl) of the antigens (CCoV and CPV), respectively. Mice from groups six, seven and eight were inoculated with the different doses of the antigens ($0.75x10^6$, $1.5x10^6$ e $3x10^6$ $TCID_{50}$, respectively) with EEP (400 µg/dose). Blood samples were collected on all animals from each group, on day of the first inoculation and 21 days after the third inoculation to measure level of the antibodies. The serum was inactivated at 56°C for 30 minutes and stored at –20°C until use.

Antibodies levels were measured by indirect ELISA as previously described[17,18], with slight modifications. The supernatants of Crandell feline kidney (CrFK) cell infected with Cornell strain of CPV (ATCC VR-2017) or strain 795 Mav of CCoV (courtesy of the Laboratory of Virology from Universidade Federal de Santa Maria - Brazil), or mock infected cultures were harvested 24 h post-infection. Cells and supernatants were frozen at -70 ºC and clarified at 250 × g for 20 min at 4 °C. Subsequently, the CPV supernatant was resuspended in carbonate buffer (Na_2CO_3 15 mM, $NaHCO_3$ 35 mM [pH 9,6]). The CCoV was centrifuged for 1 h at 140000 × g at 4 °C. The pellets were resuspended in PBS (pH 7.2) and stored at -70 °C until use.

Immunoplates were coated with CPV antigen (1:200) or CCoV (1:800) diluted in carbonate buf-

fer and incubated overnight at 4°C. The plates were washed in phosphate-buffered saline (PBS-T) containing 0,05 per cent Tween 80 (CPV) or Tween 20 (CCoV) and then treated with a blocking solution of 5 per cent of bovine foetal serum for 60 minutes at 37 °C and again washed with PBS-T. Each mice serum, diluted 1:50 in PBS-T, was added in duplicate and the plates were incubated for 60 minutes at 37 °C. After a washing cycle, peroxidase-conjugated goat anti-mice immunoglobulin IgG (Sigma Chemicals), diluted in PBS-T, was added to each well and the plates were incubated for one hour at 37 °C. After another washing cycle, freshly prepared substrates were placed into each well (ortofenil diamina - OPD), for 10 minutes. The optical density (OD) was determined at 450 nm (CPV) or 405 nm (CCoV) using an ELISA Microplate Reader (Thermo Plate – TP reader). Each sample was tested in duplicate. The adjusted OD values of each sample were obtained by subtracting the absorbance of the mock antigen-coated well from that of the corresponding virus antigen-coated well.

Statistical analysis

Antibody titers were compared using variance analysis (ANOVA) with repeated measurements. The L.S.D. test was used to determine significant differences ($P < 0.05$) among the mean of each treatment using the Statistix program.

Results

Animals in groups 1 and 2 (controls without antigen) did not produce specific antibodies (data not shown). The mean ± standard error of the mean titres obtained for CPV experimental group, in the presence or absence of green propolis is shown in figure 1. There was a significant increase in levels of IgG to CPV ($p < 0.05$) in the group inoculated with the highest concentration of antigens ($3x10^6$ $TCID_{50}$) co-ad-

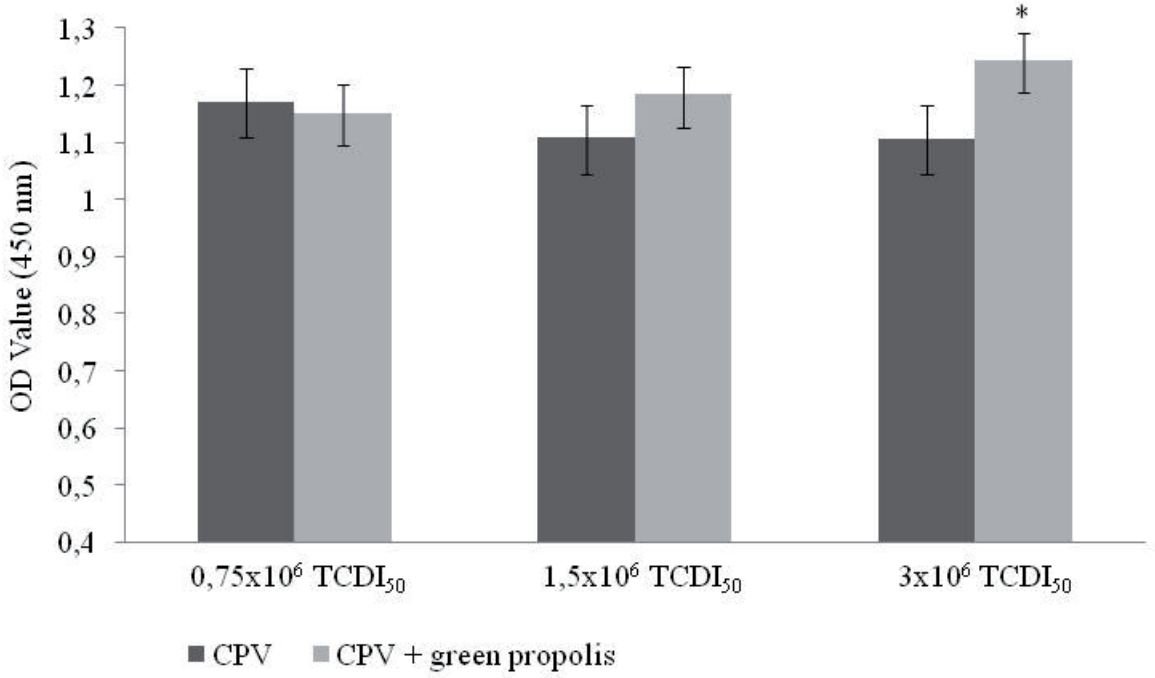

Figure 1 - Serum anti-CPV IgG levels (n=8/group) by indirect ELISA. Values represent mean ± S.D., 21 days after the third inoculation. Comparison made between groups receiving the same concentration of antigen, without green propolis and with 400 µg/dose of green propolis. Significant differences were designated as * P<0,05

ministered with 400 µg/dose of the EEP, compared to animals that received only the vaccine. There were no statistically significant differences in antibody levels to CCoV in the immunized mice in the presence or absence of green propolis (data not shown).

Discussion

The data obtained allowed the detection of different immunomodulatory properties of the EEP, depending on the type of antigen used. The addition of 400 µg/dose of this extract to the vaccine increased the potency of the humoral immune response to CPV when compared to animals that receive antigens without propolis. However, this ability was evident when the CPV was inoculated in higher dose ($3x10^6$ $TCID_{50}$). The increase in the humoral immune response, however, was not detected when evaluated to CCoV. The antibody titers in this case had no statistically significant differences in the immunized animals in the presence or absence of the EEP, indicating lack of ability of modulating the synthesis of antibodies to CCoV.

Previous studies have shown stimulant activity on the humoral immune response when there was administration of ethanol extracts of green propolis associated to antigens inactivated such as bovine and swine herpesvirus[6,7]. We note in these studies that the propolis was administered in the form of oil emulsion, together with the antigen. It is conceivable that the combination of oil with propolis allowed the formation of a deposit in the inoculation site, resulting in a slow and extended antigen and propolis release[19], allowing a constant stimulus of the immunological system. According to Cox and Coulter[19] soluble substances, such as propolis, may be rapidly processed by the cells of the immune system stimulating a weak humoral immune response. In the present study it is possible that the way in how the EEP was used (not emulsified) had not allowed the manifestation of this action on the CPV at low concentration, or on the CCoV.

The ability or lack of immunostimulating activity of the EEP may be associated with the different antigenic characteristics between these viruses. Different antigens can generate different immune responses, due to the difference in type of APCs that encounter the antigen, the density of antigen, the expression of costimulatory molecules and cytokine milieu. On the basis of cytokines profile, CD4+ T cells can be differentiated into Th1 and Th2 subtypes. Th1 cells secrete IFN-γ and other cytokines, and are mainly involved in the generation of cell-mediated immunity. Th2 cells are generally involved in humoral immunity[20]. These differentiated cells are the effector arms of the immune system that respond to different antigens. APCs, costimulatory molecules and pro-Th1 and pro-Th2 cytokines operate in a complex and concerted manner that can manipulate the activation of effector Th1 and Th2 cells[21]. In this context, it is possible that the production of antibodies in this study may have been influenced by an immunomodulatory action of propolis exerted during the initial recognition of antigens on APCs or on costimulatory molecules, stimulating different cytokines in response to each antigen, resulting in increase of Th2 response to CPV. Therefore it can be speculated that Th2 type lymphocytes were targeted by this propolis after CPV exposure. Unfortunately it was not possible to quantify expression of pro-Th1 cytokines to evaluate stimulation of cell-mediated immune response.

The precise mechanism of action of propolis on cells from the immune system is poorly understood, partly due to the chemical complexity and heterogeneous composition of propolis, resulting in controversial results[11,3]. Still the macrophages modulation should be one of the mechanisms involved[22]. The macrophage stimulation[23,24], as well as the action on production of cytokines[25] or other molecules[26] was described by other researchers. It was also recently reported that the propolis increases the *Toll-like* receptors (TLRs) expression. An increased TLR-2 expression in macro-

phages and spleen cells and increased TLR-4 expression in macrophages it was demonstrated[27]. This action may be an indirect form of immunomodulation. TLRs are widely expressed by various cells of the immune system. TLRs recognize conserved pathogen-associated molecular patterns shared by different microorganisms, such as viral RNA and DNA, among other, playing an essential role in the innate immune response and in the initiation of adaptive immune response[28]. TLRs stimulation leads to upregulation of pro-inflammatory cytokines, chemokines, antimicrobial peptides, and additional defense molecules, in addition to an increase in the processing and presentation of antigens to lymphocytes[27,28].

The immunomodulation is still a vast field for research. Given the present results we can conclude that the EEP can be a valuable resource for immunomodulation, but its activity must be assessed for each situation and particular agent. In this study the ethanolic extract of green propolis had immunomodulatory action closely dependent on the type and concentration of antigen used, being able to increase the levels of antibodies to CPV.

Acknowledgements

We are grateful to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support.

References

1. CASTALDO, S.; CAPASSO, F. Propolis: an old remedy used in modern medicine. **Fitoterapia,** v. 73, n. 1, p. S1-S6, 2002.

2. PARK, Y. K.; ALENCAR, S. M.; SCAMPARINI, A. R. P.; AGUIAR, C. L. Própolis produzida no sul do Brasil, Argentina e Uruguai: evidências fitoquímicas de sua origem vegetal. **Ciência Rural,** v. 32, n. 6, p. 723-731, 2002.

3. SFORCIN, J. M. Propolis and the immune system: a review. **Journal of Ethnopharmacology,** v. 113, n, 1, p. 1-14, 2007.

4. SFORCIN, J. M.; ORSI, R. O.; BANKOVA, V. Effect of propolis, some isolated compounds and its source plant on antibody production. **Journal of Ethnopharmacology**, v. 98, n. 3, p. 301-305, 2005.

5. CHU, W. H. Adjuvant effect of propolis on immunization by inactivated *Aeromonas hydrophila* in carp (*Carassius auratus gibelio*). **Fish and Shellfish Immunology**, v. 21, n. 1 p. 113-117, 2006.

6. FISCHER, G.; CLEFF, M. B.; DUMMER, L. A.; PAULINO, N.; PAULINO, A. S.; VILELA, C. O.; CAMPOS, F. S.; STORCH, T.; D´AVILA V. G.; DE OLIVEIRA, J. S.; VIDOR, T. Adjuvant effect of green propolis on humoral immune response of bovines immunized with bovine herpesvirus type 5. **Veterinary Immunology and Immunopathology,** v. 116, n. 1/2, p. 79-84, 2007.

7. FISCHER, G.; CONCEIÇÃO, F. R.; LEITE, F. P. L.; DUMMER, L. A.; VARGAS, G. D.; HÜBNER, S. O.; DELLAGOSTIN, O. A.; PAULINO, N.; PAULINO, A. S.; VIDOR, T. Immunomodulation produced by green propolis extract on humoral and cellular responses of mice immunized with SuHV-1. **Vaccine,** v. 25, n. 7, p. 1250-1256, 2007.

8. BANKOVA, V. S.; CASTRO, S. L. de; MARCUCCI, M. C. Propolis: recent advances in chemistry and plant origin. **Apidologie,** v. 31, n. 1, p. 3-15, 2000.

9. GALAL, A.; ABD EL-MOTAAL, A. M.; AHMED, A. M. H.; ZAKI, T. G. Productive performance and immune response of laying hens as affected by dietary propolis supplementation. **International Journal of Poultry Sciences,** v. 7, n. 3, p. 272-278, 2008.

10. BARROS, M. P.; LEMOS, M.; MAISTROB, E. L.; LEITE M. F.; SOUSA, J. P. B.; BASTOS, J. K.; ANDRADE, S. F. Evaluation of antiulcer activity of the main phenolic acids found in Brazilian Green Propolis. **Journal of Ethnopharmacology**, v. 120, n. 3, p. 372-377, 2008.

11. FISCHER, G.; HÜBNER, S. O.; VARGAS, G. D.; VIDOR, T. Imunomodulação pela própolis. **Arquivos do Instituto Biológico,** v. 75, n. 2, p. 247-253, 2008.

12. PAULINO, N.; ABREU, S. R. L.; UTO, Y.; KOYAMA, D.; NAGASAWA, H.; HORI, H.; DIRSCH, V. M.; VOLLMAR, A. M.; SCREMIN, A.; BRETZ, W. A. Anti-inflammatory effects of a bioavailable compound, Artepillin C, in Brazilian propolis. **European Journal of Pharmacology,** v. 587, p. 296-301, 2008.

13. STRASSER, A.; MAY, B.; TELTSCHER, A.; WISTRELA, E.; NIEDERMULLER, H. Immune modulation following immunization with polyvalent vaccines in dogs. **Veterinary Immunology and Immunopathology,** v. 94, n. 3/4, p. 113-121, 2003.

14. HELDENS, J. G.; PATEL, J. R.; CHANTER, N.; TEN THIJ, G. J.; GRAVENDIJCK, M.; SCHIJNS, V. E.; LANGEN, A.; SCHETTERS, T. P. Veterinary vaccine development from an industrial perspective. **The Veterinary Journal,** v. 178, n. 1, p. 7-20, 2008.

15. PAULINO, N.; SCREMIN, F. M.; RAICHASKI, L. B.; MARCUCCI, M. C.; SCREMIN, A.; CALIXTO, J. B. Mechanisms involved in the relaxant action of the ethanolic extract of propolis in the guinea-pig trachea in vitro. **Journal of Pharmacy and Pharmacology,** v. 54, n. 6, p. 1-9, 2002.

16. CONSELHO FEDERAL DE MEDICINA VETERINARIA. Resolução n^0 714, de 20 de JUNHO de 2002. **Dispões sobre procedimentos e métodos de eutanásia em animais.** Diário Oficial da União (DOU), 21 de junho de 2002. Disponível em: < http://www.cfmv.org.br/portal/legislacao/resolucoes/resolucao_714.pdf >. Acesso em: 04 de junho de 2012.

17. PHUKAN, A.; SARMA, D. K.; DEKA, D.; BORO, P. Standardization of ELISA for detection of canine parvovirus infection. **Indian Veterinary Journal**, v. 82, n. 4, p. 355-356, 2005.

18. PRATELLI, A.; ELIA, G.; MARTELLA, V.; PALMIERI, A.; CIRONE, F.; TINELLI, A. CORRENTE, M.; BUONAVOGLIA, C. Prevalence of canine coronavirus antibodies by an enzyme-linked immunosorbent assay in dogs in the south of Italy. **Journal of Virology Methods,** v. 102, n. 1/2, p. 67-71, 2002.

19. COX, J. C.; COULTER, A. R. Adjuvants – a classification and review of their modes of action. **Vaccine,** v. 15, n. 3, p. 248-256, 1997.

20. ABBAS, A. K.; MURPHY, K. M.; SHER, A. Functional diversity of helper T lymphocytes. **Nature,** v. 383, p. 787-793, 1996. doi:10.1038/383787a0.

21. SINGH, V.; AGREWALA, J. N. Regulatory role of pro-Th1 and pro-Th2 cytokines in modulating the activity of Th1 and Th2 cells when B cell and macrophages are used as antigen presenting cells. BMC **Immunology,** v. 7, p. 17, 2006. doi: 10.1186/1471-2172-7-17.

22. ANSORGE, S.; REINHOLD, D.; LENDECKEL, U. Propolis and some of its constituents down-regulate DNA synthesis and inflammatory cytokine production but induce TGF-beta 1 production of human immune cells. **Zeitschrift für Naturforsch,** v. 58, n. 7/8, p. 580-590, 2003.

23. DIMOV, V.; IVANOVSKA, N.; MANOLOVA, N.; BANKOVA, V.; NIKOLOV, N.; POPOV, S. Immunomodulatory action of propolis. Influence on anti-infections protection and macrophage function. **Apidologie,** v. 22, n. 2, p. 155-162, 1991.

24. SFORCIN, J. M.; KANERO, R.; FUNARI, S. R. C. Absence of seasonal effect on the immunomodulatory action of brazilian propolis on natural killer activity. **The Journal of Venomous Animals and Toxins,** v. 8, n. 1, p. 19-29, 2002.

25. HAN, S.; SUNG, K. H.; YIM, D.; LEE, S.; CHO, K.; LEE, C. K.; .; KIM, K. Activation of murine macrophage cell line RAW 264.7 by Korean propolis. **Archives of Pharmacol Research,** v. 25, n. 6, p. 895-902, 2002.

26. KRÓL, W.; CZUBA, Z. P.; THREADGILL, M. D.; CUNNINGHAM, B. D. M.; SHANI, J. Modulation of luminol-dependent chemiluminescence of murine macrophages by flavone and its synthetic derivatives. **Drug Research,** v. 45, n. 7, p. 815-818, 1995.

27. ORSATTI, C. L.; MISSIMA, F.; PAGLIARONE, A. C.; BACHIEGA, T. F.; BUFALO, M. C.; ARAÚJO JR., J. R.; SFORCIN, J. M. Propolis immunomodulatory action *In Vivo* on Toll-like receptors 2 and 4 expression and on pro-inflammatory cytokines production in mice. **Phytotherapy Research,** v. 24, n. 8, p. 1141-1146, 2010.

28. MEDZHITOV, R. Toll-like receptors and innate immunity. **Nature Revews Immunology,** v. 1, n. 2, p. 135-145, 2001.