The effects of propolis on antibody production by laying hens

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ABSTRACT Propolis is a honeybee product showing several biological properties that enhance the immune response, depending on the concentration and intake period. Because propolis possesses an immunomodulatory action on mammals, the objective of our study was to investigate the effects of propolis on the humoral immune response of laying hens by evaluating antibody production. Laying hens (ISA Brown) were divided into 5 groups with 7 birds each. Group 1 was a nonimmunized control, whereas birds in group 2 were immunized intravenously with SRBC, and those in groups 3, 4, and 5 were treated intraperitoneally with propolis (2, 10, and 50 mg/kg, respectively) on 3 consecutive days and then inoculated intravenously with SRBC. Hematological and serological analyses were carried out on d 0, 3, and 38. Natural and specific antibody levels were determined by hemagglutination with rabbit red blood cells and SRBC, respectively. Propolis-treated birds (50 mg/kg) showed a significant decline in heterophils and in the heterophil:lymphocyte ratio. After SRBC immunization, significant increases in levels of IgG were observed in groups 4 and 5. Furthermore, higher levels of natural antibodies were observed in propolis-treated laying hens. The administration of propolis to laying hens increased the production of IgG specific to SRBC and natural antibodies, and could be used to increase antigen-specific antibody responses to vaccines.

Key words: antibody, humoral immune response, laying hen, propolis

INTRODUCTION

Propolis is a resinous material that honeybees collect from plants and mix with wax and salivary enzymes. Its immunomodulatory action has been widely investigated in vivo and in vitro in recent decades (Sforcin, 2007).

Previous works in our laboratory showed that administration of propolis to mice for 3 d increased proinflammatory [interleukin (IL)-1α and IL-6] cytokine production and stimulated the expression of toll-like receptor-2 and toll-like receptor-4), suggesting that propolis may activate the initial steps of the immune response (Orsatti et al., 2010a). In this same model, we analyzed the expression and production of Th1 (IL-2 and interferon-γ) and Th2 (IL-4 and IL-10) cytokines, and verified that short-term propolis administration to mice affected interferon-γ production, which may be related to its anti-inflammatory properties (Orsatti et al., 2010b). These data showed that propolis could exert both pro- and anti-inflammatory effects, depending on concentration, intake period, and route of administration (Sforcin, 2007).

With regard to the humoral response, propolis and some of its isolated constituents stimulate antibody production. An increase in antibody production was observed after the administration of Ethanolic extract of propolis to mice (Scheller et al., 1988). A similar effect was detected in propolis-treated rats immunized with BSA (Sforcin et al., 2005) as well as in cattle immunized with inactivated bovine herpesvirus type 5 vaccine (Fischer et al., 2007).

Flavone (70.99% rutin), a component of propolis associated with epimedium polysaccharide (71.23% glucose), had a potent adjuvant action in rabbits inoculated with inactivated rabbit hemorrhagic disease virus (Yang et al., 2008). In carp, administration of propolis simultaneously with an inactivated vaccine against Aeromonas hydrophila also increased antibody titers (Chu, 2006). However, there are few studies regarding propolis activity on the humoral immunity of chickens. In broilers, dietary supplementation with oil-extracted propolis induced higher titers of antibodies to avian influenza, Newcastle disease, and infectious bursal disease vaccines compared with those of nontreated birds.
Increased antibody levels to Newcastle disease vaccine were found in chickens when the vaccine was mixed with a Chinese herbal medicine containing propolis flavone and epimedium polysaccharide (Wang et al., 2006). Furthermore, increased antibody production was seen when propolis polysaccharide was inoculated subcutaneously for 3 consecutive days in chickens immunized with Newcastle disease vaccine (Kong et al., 2006). On the other hand, caffeic acid and quercetin isolated from propolis did not seem to affect antibody production in rats (Sforcin et al., 2005).

Recently, Sun et al. (2008) verified that the adjuvant action of propolis on the immune response of chickens to F4 fimbriae led to an enhanced antibody titer compared with F4 antigen alone. Herein, we report the effects of the ethanolic extract of propolis as an immunomodulatory agent on the humoral immunity of laying hens.

**MATERIALS AND METHODS**

**Propolis Hydroalcoholic Solution**

Propolis was collected in the Beekeeping Section of the School of Veterinary Medicine and Animal Husbandry, Universidade Estadual Paulista, from a hive of cultivated Africanized honeybees (*Apis mellifera* L.) by using plastic nets. After a month, the nets were taken and frozen to promote propolis removal. The propolis was ground and a 30% ethanolic extract was prepared in sterile conditions (30 g of propolis completing the volume to 100 mL with 70% ethanol), in the absence of bright light, at room temperature and was shaken moderately. After a week, the extracts were filtered and final concentrations were calculated to obtain the dry weight of the solutions (120 mg/mL). Chemical composition of propolis was investigated by thin-layer chromatography, gas chromatography, and gas chromatography-mass spectrometry analysis (Boudourova-Krasteva et al., 1997; Bankova et al., 1998a,b). This sample was frozen and has been used until now to prepare new extracts.

**Chickens**

Laying hens (ISA Brown) weighing approximately 2 kg (2.027 ± 0.029) and aged 20 wk were obtained from the School Farm of the Universidade Estadual de Londrina (UEL), and were kept in individual cages for a week at room temperature before the experiments were conducted. Birds were subjected to a cycle of 17 h of light, and received water and a laying hen diet (Purina, Brazil) ad libitum throughout the experimental period.

**Experimental Design**

Laying hens were randomly divided into 5 groups of 7 birds each, as follows: group 1—control; group 2—birds were immunized intravenously with SRBC; group 3—birds were treated intraperitoneally with propolis (2 mg/kg) and inoculated intravenously with SRBC; group 4—birds were treated with propolis (10 mg/kg) + SRBC; group 5—birds were treated with propolis (50 mg/kg) + SRBC.

Initially (d 0), blood samples were collected from all birds for hematological analysis and plasma collection. On the first day of the experiment (d 1), birds from groups 1 and 2 received only the vehicle (0.15 M PBS, pH 7.2), whereas birds from groups 3, 4, and 5 were inoculated intra-abdominally with 2, 10, and 50 mg/kg of propolis in vehicle (0.15 M PBS, pH 7.2), respectively. Inoculations were repeated on d 2 and 3 in the morning.

In the afternoon of d 3, laying hens were bled and birds from groups 2, 3, 4, and 5 were inoculated intravenously with 5% SRBC in 0.15 M PBS, pH 7.2. Birds in group 1 were inoculated with only the vehicle (0.15 M PBS, pH 7.2). On d 31, birds from groups 2, 3, 4, and 5 were inoculated intravenously with 5% SRBC, whereas birds from group 1 were injected with only the vehicle (0.15 M PBS, pH 7.2). A new blood sample was taken on d 38 for hematological and antibody analysis. Body weights of the chickens were recorded on d 1 and 38.

**Hematological Analysis**

Blood samples were collected by brachial venipuncture according to the method of Zander and Mallinson (1991). After collection, blood was placed in glass tubes with anticoagulant solution (5% EDTA, Newprov, Pinhais, Brazil). Blood smears were prepared and stained with a dye solution of May-Grunwald Giemsa (Laborclin, Pinhais, Brazil; Jain, 1986). The complete blood count of peripheral cells was done according to the method of Silveira et al. (2009). Total protein concentration was obtained by refractometry (Cray et al., 2008).

**Antibody Production**

Anti-SRBC antibody production was determined by hemagglutination (Sandhu et al., 2007). Briefly, sheep blood was collected (vol/vol) in Alservier’s solution. Sheep red blood cells were washed 3 times with 0.15 M PBS, pH 7.2, and resuspended at a concentration of 2% in 0.15 M PBS, pH 7.2. All plasma samples were inactivated at 56°C for 30 min before use. Plasma samples were serially diluted (vol/vol, dilution factor of 2) in 25 μL of 0.15 M PBS, pH 7.2, in U-bottomed microtiter plates (Kartell, Noviglio, Italy). Afterward, 25 μL of SRBC at a concentration of 2% was added and the reaction was incubated for 2 h at 37°C and for 22 h at 4°C. Plates were read after 24 h to compare the patterns of sedimentation of the control well without plasma with wells containing serial dilutions of the plasma. The antibody titer was read as the highest dilution that showed a positive agglutination of SRBC.
For determination of the antibodies resistant to 2-mercaptoethanol, samples were incubated (vol/vol) with 0.2 M 2-mercaptoethanol (Vetec, Rio de Janeiro, Brazil) in PBS, pH 7.2, for 30 min at 37°C and were used for hemagglutination as described above. The results were expressed as the log of the reciprocal dilution corresponding to the antibody titer. The natural antibody titers were determined as described above, but using rabbit red blood cells (RRBC) instead of SRBC.

**Statistical Analysis**

Antibody titers, hematological parameters, and BW were analyzed by one-way ANOVA for the effect of propolis treatment and to compare the groups at different periods of time. A P-value of 0.05 was chosen as the significance level. One-way ANOVA with Tukey’s post-test was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

**RESULTS**

**Constituents of the Propolis Hydroalcoholic Solution**

Because propolis composition may vary according to the local flora, we used a chemically characterized sample. The main constituents of our propolis sample were identified by thin-layer chromatography, gas chromatography, and gas chromatography-mass spectrometry: flavonoids (kaempferide, 5,6,7-trihydroxy-3,4′-dimethoxyflavone, aromadendrine-4′-methyl ether); essential oils [spathulenol, (2Z,6E)-farnesol, benzyl benzoate, and prenylated acetophenones]; a prenylated p-coumaric acid and 2 benzopyranes (E and Z 2,2-dimethyl-6-carboxyethenyl-8-prenyl-2H-benzopyranes); 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); and di- and triterpenes, among others (Boudourova-Krasteva et al., 1997; Bankova et al., 1998a,b).

**Effect of Propolis on Hematological Parameters**

The effects of propolis treatment on the hematological parameters of laying hens are shown in Figure 1. The hematocrit values showed no significant changes among the groups. Increased total leukocytes were seen in all groups, including the control group, after inoculation with propolis (d 3), but this increase was significant only in group 2 (antigen), group 3 (2 mg/kg), and group 5 (50 mg/kg). Furthermore, a significant increase in total leukocytes was observed after the second antigen administration (d 38) compared with the initial period of the experiment in group 4 (10 mg/kg) and group 5 (50 mg/kg).

With respect to the levels of heterophils, a significant increase was observed after d 3 in groups 2 and 5 and after immunization with SRBC in group 3. Furthermore, a significant difference was observed between groups 3 and 5 after inoculation with propolis, and lower levels of heterophils were observed after immunization with SRBC in group 5.

The levels of total lymphocytes increased significantly after d 3 in groups 1 and 4. Furthermore, an increase in the number of lymphocytes was seen in group 3 only after propolis inoculation, whereas group 5 showed higher levels of lymphocytes after SRBC inoculation.

With regard to the heterophil:lymphocyte ratio, an increase was observed in group 5 after propolis inoculation, followed by a significant reduction after inoculation with SRBC. A significant reduction in total protein levels was seen in group 5 laying hens after propolis inoculation, followed by a significant increase after SRBC inoculation.

**Antibody Titers**

Titers of anti-SRBC blood cells in the experimental groups are presented in Figure 2. All birds showed similar levels of antibodies against sheep erythrocytes at the beginning of the experiment. After propolis administration (d 3), there was an increase, although not significant, in antibody levels in all groups. Moreover, a significant difference was observed in total and IgM antibodies between groups 2 and 3.

As expected, the levels of total antibodies in the secondary response (d 38) increased significantly in all groups when compared with those in group 1. This increase was caused by a significant increase in levels of IgG, but not IgM. In addition, a significant increase in the levels of IgG antibodies was observed in sera from birds in groups 4 and 5 compared with those in group 2.

With regard to levels of natural antibodies detected by hemagglutination of RRBC, no significant differences were observed between groups. In addition, no significant changes in antibody levels were observed after propolis treatment. However, after inoculation of SRBC, increased levels of total anti-RRBC antibodies were observed in groups 3, 4, and 5 compared with the values before treatment. This increase was due to a significant increase in levels of IgM antibodies in groups 3 and 4 (Figure 3).

**BW**

No significant difference was observed in the average BW of birds in the experimental group compared with those in the control group.

**DISCUSSION**

The aim of this study was to investigate the effects of propolis on antibody production by laying hens because
Propolis has been shown to exert a potent action on the immune system (Sforcin, 2007). Our work evaluated the possible immunomodulatory action of propolis on anti-SRBC production in laying hens. The inoculation of ethanolic extract of propolis before SRBC immunization had a positive effect on the IgG-mediated response because a significant increase in IgG levels in response to SRBC was observed in birds treated with 10 and 50 mg/kg of propolis. This effect appears to be dose-dependent because the birds treated with 2 mg/kg of propolis showed increased levels of IgG, but these were not significant. Furthermore, the propolis treatment had no effect on IgM titers. These results are in agreement with other studies reporting the immunostimulatory action of propolis (Kong et al., 2004, 2006; Taheri et al., 2005; Wang et al., 2005; Cetin et al., 2010).

Regarding the humoral immune response, studies have shown that propolis is able to increase antibody production in SRBC-immunized mice (Scheller et al., 1988). In birds, few studies have investigated the immunostimulatory effect of propolis (Kong et al., 2004, 2006; Taheri et al., 2005; Wang et al., 2005; Galal et al., 2008). Recently, a comparative study of the adjuvant effect of propolis extract, Cochichina momordica seed, Quil A, and Freund’s adjuvant on the production of anti-F4 fimbrial antigen in laying hens showed that propolis had an adjuvant effect, but this effect was less intense than that of other adjuvants (Sun et al., 2008). The ability of propolis to modulate the adaptive im-

**Figure 1.** Hematological values of propolis-treated laying hens immunized with SRBC. Values are expressed in mean ± SEM of 7 birds. *a* significantly different from d 0; *b* significantly different from d 3; *c* significantly different from group 5 (*P* < 0.05). G1, G2, G3, G4, and G5 = groups 1 to 5, respectively.
mune response is probably due to a potent stimulatory effect on different cells of the innate immune response, such as macrophages and natural killer cells (Sforcin, 2007). These cells influence adaptive immunity by secreting cytokines that modulate the function of T and B cells (Sforcin et al., 2002; Orsi et al. 2005; Orsatti et al., 2010a). A possible mechanism involved in the increased levels of antibodies produced after antigenic stimulation in propolis-treated birds is probably related to increased expression of IL-2 and interferon-γ because these cytokines stimulate antibody production in birds (Wang et al., 2006).

Natural antibodies are found in nonimmunized birds; in mammals, natural antibodies are usually IgM with a low affinity for several antigens (Casali and Notkins, 1989; Boes, 2000) and are related to initial protection against infections, enhanced cellular and humoral immune responses, and prevention of autoimmune diseases (Avrameas, 1991; Ochsenbein et al., 1999; Ochsenbein and Zinkernagel, 2000; Stäger et al., 2003). Passive adoptive transfer experiments have shown their involvement in regulating the specific immunity response to antigens (Lammers et al., 2004), and that natural antibody titers are correlated with chicken survival (Star et al., 2007). Furthermore, high natural antibody titers to a specific antigen may depress the humoral immune response to the same antigen (Parmentier et al., 2008). Natural antibody levels may be genetically related to specific antibody levels, as suggested by the higher levels of natural antibodies found in chickens lines selected for high antibody levels, but not in those selected for low antibody levels (Parmentier et al., 2004). In chickens, natural antibodies are easily detected by RRBC agglutination, probably because of the Galα1–3Galβ1–4GlcNAc antigen (Cotter et al., 2005).

In our study, propolis treatment had no significant effects on natural antibody levels against RRBC. On the other hand, natural antibody levels were increased in propolis-treated groups inoculated with SRBC compared with the initial antibody levels. This response was caused by IgM, but not by IgG, as expected for cross-reactive antibodies specific to SRBC, and IgM was produced in significantly amounts by birds inoculated with SRBC. Furthermore, laying hens not treated with propolis but immunized with SRBC did not have increased IgM levels. Thus, the higher level of natural IgM in chickens may be caused by an immunostimulatory effect of propolis and not by a specific response to SRBC. Although the mechanisms of action of propolis on the immune system of mammals have been investigated in recent years (Sforcin, 2007), further studies are necessary to investigate the effects of propolis on natural antibody titers in chickens.

Propolis appears to have a significant effect on the hematological parameters of birds. Laying hens supplemented with 100 and 150 mg/kg of propolis in the diet had significantly higher hematocrit and total protein levels compared with those of the control group (Ga-lal et al., 2008). Furthermore, medium and high levels

Figure 2. Effect of propolis on specific antibody titers to SRBC. Means ± SEM of SRBC antibody titers expressed as log 2 reciprocal to the highest dilution with visible agglutination.  *Significantly different from the secondary antibody titers of group 1;  #significantly different from group 2. G1, G2, G3, G4, and G5 = groups 1 to 5, respectively.
of propolis supplementation significantly reduced heterophils and increased lymphocyte numbers compared with the control group (Galal et al., 2008). In our study, increases in total levels of leukocytes, heterophils, and lymphocytes were observed after inoculation of propolis and after immunization with SRBC. This result may be due to the stress of birds when they were restrained for blood collection and inoculation of propolis and antigen. Furthermore, increases in heterophils and in the heterophil:lymphocyte ratio were observed in group 5 after inoculation with propolis, suggesting a stimulatory action of propolis on heterophil production. A possible inflammatory effect of propolis (50 mg/kg) in the abdominal cavity cannot be discounted.

In summary, the administration of propolis to laying hens increased the production of IgG specific to SRBC and natural IgM antibodies, and it altered hematological parameters. These data are important because propolis could be used to enhance antigen-specific antibody production in laying hens, suggesting its use as an adjuvant to antibody production in mammals as well (Sforcin, 2007). Furthermore, the increased production of antibodies suggests that propolis has an immunostimulatory effect on chickens and could be used to increase antigen-specific antibody responses to vaccines. Further studies are necessary to investigate whether propolis enhances the production of antibodies to other antigens and if it could protect birds against disease challenge.

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REFERENCES


