Immune Activation and Radioprotection by *Propolis*

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Abstract: In this study, we focused on immune stimulation by *Propolis*, and examined changes in the effect of irradiation after *Propolis* administration. We also examined the radioprotective effect of *Propolis* by observing its effect on the immune system. The effect of immune activation by *Propolis* was investigated by measuring the total immunoglobulin (Ig) G and IgM. The radioprotective effect of immune activation by *Propolis* was investigated by measuring the T-lymphocyte subsets in the peripheral blood of mice following whole body irradiation.

Compared with the control group, the IgG was significantly reduced in the *Propolis* group, indicating that *Propolis* suppressed IgG production. ELISA revealed that the amount of IgM in mouse serum was significantly higher in the *Propolis* group as compared with the control group, indicating that *Propolis* increased IgM production.

The number of CD4-positive cells was increased only in the *Propolis* group. Likewise, the number of CD4-positive cells increased by 81% in the *Propolis* with irradiation group compared with the irradiation group alone. Compared with the control group, the *Propolis* group increased CD8-positive cells. Compared with the irradiation alone group, CD8-positive cells were decreased by *Propolis* with irradiation group.

Propolis activated macrophages to stimulate interferon (IFN)-γ production in association with the secondary activation of T-lymphocytes, resulting in a decrease in IgG and IgM production. Cytokines released from macrophages in mouse peripheral blood after *Propolis* administration activated helper T-cells to proliferate. In addition, activated macrophages in association with the secondary T-lymphocyte activation increased IFN-γ production and stimulated proliferation of cytotoxic T-cells and suppressor T-cells, indicating the activation of cell-mediated immune responses.

Keywords: *Propolis*; Radiation Protection; T-Lymphocyte; IgG; IgM; CD4; CD8.

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Introduction

Propolis, a resinous product collected by honeybees from plants, has been used as folk medicine since ancient times (Kong *et al*., 2004; Melliou and Chinou, 2004; Ilhan *et al*., 2004). The immunoregulatory and anti-inflammatory properties of *Propolis* have been published (Harish *et al*., 1997; Blonska *et al*., 2004) recently. However, the detailed mechanisms of *Propolis* and its components on immune cells are still unknown (Ansorge *et al*., 2003). Therefore, we studied the effects of different *Propolis* extracts, the flavonoids hesperidin, quercetin, as well as caffeic acid phenethyl ester (CAPE) on basic mouse immune cell functions. Radiation has tremendous therapeutic benefits for humans; however, it is also associated with the risk of serious adverse effects. Examples of radiation-protective agents that have been tested clinically include: SH compounds, such as cysteine and WR-2721 (amifostine), which remove radicals produced by radiation and thereby protect the body from the indirect effects of radiation (Georgieva *et al*., 2002; Andeassen *et al*., 2003); granulocyte colony-stimulating factor (G-CSF), which prevents immunosuppression from radiation exposure; and anti-immunosuppressives, such as OK-432 (Yang *et al*., 2003; Jorgensen *et al*., 2003). However, these medications have the potential to cause serious adverse effects, particularly when combined with other medications. With this limitation in mind, new medications derived from naturally occurring materials, which have fewer side effects and greater radiation protective potential, have been studied and developed. Recently, many traditional medicinal herbs have been re-evaluated as therapeutic agents. Several medicinal herbs have been shown to have beneficial pharmacological and physiological effects. Since they may have less harmful direct effects and side effects than other medicines, there has been increasing interest in and demand for such products (Rininger *et al*., 2000; Beuscher *et al*., 1994).

We focused on hematocytes as a marker to evaluate the protective effect of *Propolis* against irradiation and examined blood cell counts over time and antioxidant activity in the peripheral blood. We found that *Propolis* administration inhibited the reduction of white blood cell counts, especially lymphocytes and monocytes, after exposure to radiation. Therefore, we investigated the radioprotective effect of *Propolis* by measuring total immunoglobulin (Ig) G, IgM and T-lymphocyte subsets in the peripheral blood.

Material and Methods

Propolis

Propolis is a resinous hive product collected by honeybees from various plant sources (Banskota *et al*., 1998). It is extensively used in food and beverages and in folk medicine for the treatment of different ailments. It is reported that *Propolis* has a broad spectrum of pharmacological activities, such as anti-microbial activity, antioxidant, anticancer and as an immune stimulant, in addition to other pharmacological effects (Burdock, 1998). The chemical composition of *Propolis* has turned out to be very complex, with more than 200 compounds isolated so far (Burdock, 1998). The most important constituent appears to be

phenolics, which form more than 50% of the propolis composition (Bankova *et al.*, 1996). Since isolation of bioactive substances is accompanied with many difficulties owing to their complexity and scarce amounts, we devised a method to obtain the aromatic compounds by using b-cyclodextrininclusion. Preparation of the extract from the *Propolis* sample, which was collected in Brazil, and the ethanolic extract was prepared as previously described (Bankova *et al.*, 1999). Briefly, the resin was cut into small pieces and extracted with 70% ethanol, 1:10 (1:10 w/v) at room temperature for 24 hours. The extract was evaporated to dryness under a vacuum (yield 62%) and a stock solution was prepared in dimethysulfoxide (DMSO). Before use, the extract was diluted in a phosphate-buffered solution (PBS), and the final concentration of DMSO in this solution did not exceed 1%, which had no effect *per se* on animal tests. Therefore, we used the above-mentioned *Propolis* in this study.

Experimental Animals

Male C3H/HeNCrj mice (6 weeks old, 18–24 g), purchased from Charles River, Japan, were used for the studies on IgG and IgM in peripheral blood. Male C57BL/6crSlc mice (3 weeks old, 8–13 g on average, purchased from Japan SLC Inc., were used for T-lymphocyte studies. The mice were housed under conventional conditions (room temperature, $22 \pm 3°C$; humidity, 60%) with commonly available chow (CA-1, CLEA Japan Inc.) and tap water. Mice were acclimated for 1 week before the experiments.

Irradiation

Mice were systemically irradiated (2 Gy) using an x-ray generator designed for animal use (Phillips, Inc.). A plastic jig was used to restrain and rotate the mice at a constant speed so that they were irradiated evenly. The irradiation conditions were 200 kV, 0.35 Gy/min with a filter for 0.1 mm Cu + 1 mm Al.

Blood Collection

Blood was collected from the mouse by puncturing the heart with a 23-G needle under anesthesia, mixed with heparin, and either centrifuged (15 minutes at 1500 rpm) to separate serum for the immunoglobulin studies or suspended 1:1 in phosphate buffered solution (PBS), and then processed as described below for the T-lymphocyte studies.

Measurement of Total IgG and IgM in Mouse Peripheral Blood

Mice were divided into two groups: the control group, to which physiologic saline was given, and the IgG and IgM groups, to which *Propolis* was given. Each group comprised of ten mice. Total serum IgG and IgM were measured by enzyme-linked immunosorbent assay (ELISA) with a mouse IgG ELISA quantification kit and a mouse IgM ELISA quantification kit, respectively (Bethyl Laboratories Inc., Montgomery, TX).

In a 96-well microplate, 100 μl of a solid-phase antibody (affinity purified goat anti-mouse IgG-Fc or IgM antibody), diluted 100-fold with a coating buffer (0.05 M sodium carbonate, pH 9.6), was dispensed into each well and incubated at room temperature for 60 minutes. After incubation, the coating buffer was discarded and the wells were rinsed twice with washing buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0). Then, 200 μl of postcoat solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) was poured into each well to solidify the solid-phase antibody. After incubation at room temperature for 30 minutes, the postcoat solution was discarded and the wells were rinsed twice with washing buffer. Serum (100 μl) was diluted 50-fold with the sample diluting solution (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0) or known concentrations of standard serum (mouse IgG or IgM reference serum) were dispensed into the wells and incubated at room temperature for 60 minutes. After incubation, the serum was discarded, and the wells were washed four times with washing buffer. In each well, 100 μl of enzyme-labeled antibody (HRP-conjugated goat anti-mouse IgG-Fc or HRP-conjugated goat anti-mouse IgM antibodies), diluted at 1:120,000 with conjugate diluting solution (same composition as the sample diluting solution), was dispensed for enzymatic reaction. After incubation for 60 minutes, the enzyme solution was decanted and the wells were washed four times.

After rinsing, 100 μl of enzyme substrate solution (TMB; 3, 3, 5, 5-tetramethyl benzidine) was dispensed and incubated for 15 minutes to develop color. After 100 μl of stop solution (2 M H_2SO_4) was dispensed into each well, optical density (OD) was measured on a MPR A4 microplate reader (Toyosohtatsu, Japan) at a reference wavelength of 450 nm. Total serum IgG and IgM concentrations were measured after a standard curve with the ODs of standard sera was drawn. For statistical analysis, a parametric t-test was used to compare the two groups, because the concentrations of total serum IgG and IgM showed normal distributions.

Measurement of CD3-, CD4- and CD8-positive T-Lymphocytes in Peripheral Blood

Lymphocytes were separated by the gravity centrifugation method (Kristensen *et al.*, 2004). Lymphocyte separating solution (5 ml sodium hypaque, Ficoll 400; specific gravity, 1.0875 ± 0.0005 at 25°C) was added into a 15 ml sample tube, on to which 5 ml of cell suspension was carefully loaded. After centrifugation at room temperature (15–20°C) at 500 g for 20 minutes, plasma in the supernatant was collected to extract lymphocyte subsets. After the addition of PBS (pH 7.2, without Ca^{2+} or Mg^{2+}) supplemented with 10% inactivated fetal bovine serum (FBS) (heat-inactivated at 56°C for 30 minutes), and red blood cell lysing solution, the mixture was centrifuged at room temperature at 400 g for 10 minutes. The supernatant was collected and the cells were resuspended and washed twice in PBS containing FBS. Lymphocytes were resuspended in PBS prior to analysis. Flow cytometry reagents for lymphocyte subset measurement were added into the lymphocyte suspension in PBS, and the mixture was stained for immunofluorescence for about 30 minutes at 4°C in a dark room. After the reaction, the solution was rinsed three times with PBS, and CD3, CD4 and CD8 subsets were analyzed by a FACS Caliber flow cytometer (Becton Dickinson).

To analyze T-lymphocyte subsets, Multicolor Flowcytometry (FCS) System (Santa Cruz Biotechnology Inc.) was employed and CD3-, CD4-, and CD8-positive T-lymphocytes in the peripheral blood were counted by three-color flow cytometry using anti CD3-PE-Cy5.5, anti CD4-FITC and anti CD8-PE.

Statistical Analysis

Following analysis of variance, significance of the difference in each parameter among groups was assessed by t-test and the Dunnett comparison test. Values of $p < 0.05$ were considered significant.

Results

Total IgG and IgM in Mouse Peripheral Blood

A standard curve was generated based on the OD of mouse IgG standard solutions of known concentrations, and the total IgG in mouse serum was measured as shown in Table 1. Compared with the control group, total serum IgG was significantly reduced in the *Propolis* group ($p < 0.05$), indicating that *Propolis* suppressed IgG production. Likewise, a standard curve based on the ODs of mouse IgM standard sera of known concentrations was used to quantify IgM in mouse serum, as shown in Table 1. ELISA revealed that the amount of IgM in mouse serum was significantly higher in the *Propolis* group as compared with the control group ($p < 0.05$), indicating that *Propolis* increased IgM production.

Analysis of T-Lymphocyte Subsets in Mouse Peripheral Blood

On the cytogram, the lymphocyte fraction was gated, so that CD4-positive ($CD3^+CD4^+$) and CD8-positive ($CD3^+CD8^+$) cells were counted.

First, CD4-positive cells were counted by flow cytometry for comparison. As shown in Fig. 1, the number of CD4-positive cells increased by 105% in only the *Propolis* group. Likewise, the number of CD4-positive cells increased by 81% in the *Propolis* with irradiation group compared with the irradiation alone group. This indicates that *Propolis* administration increased CD4-positive cells, i.e. helper T-cells in the peripheral blood. CD8-positive cells were also counted by flow cytometry. As shown in Fig. 2, CD8-positive cells were increased

Table 1. Effect of *Propolis* on Total IgG and IgM in Mouse Serum

Groups	Total IgG (ng/ml) Mean ± SEM	IgM (ng/ml) Mean ± SEM
Control	368 ± 22.6	732 ± 36.8
Propolis	*312 ± 31.2	*812 ± 47.3

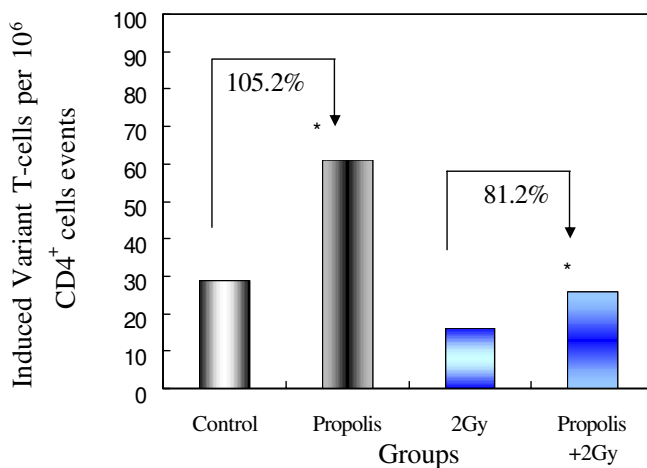*Significant difference ($p < 0.05$) between control group and *Propolis* group by t-test.

Figure 1. Comparison of the induced frequency of CD4+ in C57BL/6CrSlc. *Significant difference (p < 0.05) between control group versus *Propolis* group and 2Gy group versus *Propolis* + 2Gy group by Dunnett test.
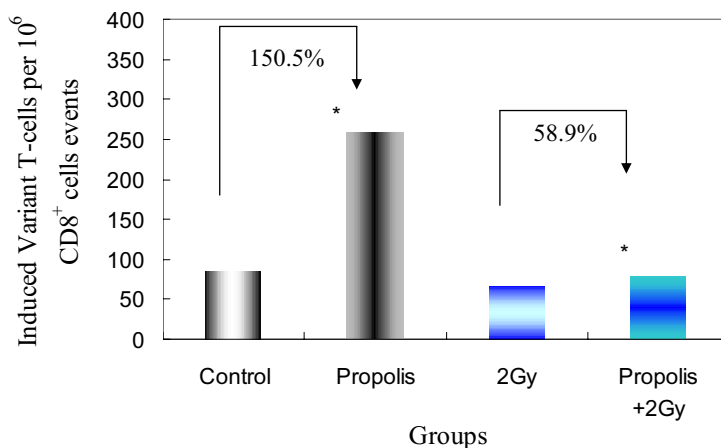

Figure 2. Comparison of the induced frequency of CD8+ in C57BL/6CrSl. *Significant difference (p < 0.05) between control group versus *Propolis* group and 2Gy group versus *Propolis* + 2Gy group by Dunnett test.

by 150% in the *Propolis* group compared with the control group. Compared with the irradiation alone group, CD8-positive cells were decreased by 58% in the *Propolis* with irradiation group. This indicated that *Propolis* administration increased CD8-positive cells, i.e. suppresser T-cells and killer T-cells in the peripheral blood. However, in the *Propolis* with irradiation group, irradiation decreased the number of CD8-positive cells.

Discussion

Total IgG and IgM in Mouse Peripheral Blood

When irradiation causes death, infection is often the direct cause. Vulnerability to infection in subjects with weakened immune systems is largely due to the impairment of lymphocytes. Since lymphocytes are extremely sensitive to irradiation, irradiation remarkably weakens the immune system. In this study, therefore, we examined the effect of *Propolis* on the production of IgG and IgM, which are responsible for the primary and secondary immune responses.

The production of IgG and IgM after *Propolis* administration was examined. *Propolis* administration decreased the production of IgG in mouse peripheral blood. However, *Propolis* administration increased the production of IgM in mouse peripheral blood.

Rehman *et al*. (1999) reported that the medicinal plants of *Echinacea angustifolia* and *Hydrastis canadensis* administered for 6 weeks increased IgG production in the early to middle term in rats. However, in contrast, *Propolis* decreased IgG production in the present study. *Propolis* has an interferon (IFN)-like activity to induce and activate macrophages and T-lymphocytes, which may explain the difference between the two results (Bousquet *et al*., 1982; Rininger *et al*., 2000). Activated macrophages secrete cytokines (IL-1, IL-6, IL-12 and TNF-α), and activate T-cells. Furthermore, activated T-cells secrete IFN-γ which inhibits differentiation to antibody-producing B cells. Suzuki *et al.* (2002) reported that *Propolis* increased IFN-γ. Therefore, the cell-mediated immune response is activated, while the humoral immune response is in turn suppressed (Paganelli *et al*., 1998; Abbas *et al.*, 1996). As observed in this study, the IFN-like activity of *Propolis* stimulated the cell-mediated immune response of macrophages and T-cells, while it suppressed the humoral immune response and reduced antibody production.

Analysis of T-Lymphocyte Subsets in Mouse Peripheral Blood

Propolis administration significantly decreased the total IgG in mouse peripheral blood. *Propolis* stimulated the cell-mediated immune response by increasing the levels of IFN-γ, which are associated with activation of macrophages and T-cells, while impairing the humoral immune response. To analyze T-lymphocyte subsets that play a central role in cell-mediated immune responses in mouse peripheral blood after *Propolis* administration, CD4 and CD8 cell surface antigens were examined by flow cytometry and the effect of *Propolis* on cell-mediated immune response was examined.

Cell-specific markers, designated as CD markers, exist on the surface of lymphocytes, and are used to sort out the "hallmark" or characteristics and function of protein in the process of differentiation of lymphocytes produced in the bone marrow to a variety of cells (Kristensen *et al*., 2004). CD3 is exclusively found in T-cells. CD4-positive (CD3^{+}, CD4^{+}) cells are helper T-cells, while CD8-positive (CD3^{+}, CD8^{+}) cells are cytotoxic T-cells and suppresser T-cells. T-lymphocyte subsets can be examined by measuring these CD markers.

In this study, CD4-positive T-lymphocytes were counted in mouse peripheral blood in each group. It was observed that *Propolis* increased helper T-cells in mouse peripheral blood. It seemed that *Propolis* activated macrophages and subsequently stimulated T-cells (Rininger *et al.*, 2000; Mosmann and Sad, 1996). Rininger *et al.* and Beuscher *et al.* reported that Propolis administration activated macrophages to increase the production of cytokines (IL-1, IL-8, IL-12 and TNF-α) (Rininger *et al.*, 2000; Beuscher *et al.*, 1994*)*. IL-1 and IL-12 can promote the proliferation of helper T-cells. This also suggests that cytokines (IL-1, IL-6, IL-12 and TNF-α) released from macrophages by *Propolis* administration activated helper T-cells to proliferate.

CD-8 positive cells in mouse peripheral blood were examined in each group of mice. It was observed that *Propolis* increased the number of cytotoxic T-cells and suppressor T-cells. The mechanism likely involved an increase in INF-γ levels following treatment with *Propolis*. The increase in IFN-γ levels, in addition to the suppression of antibody production mentioned previously, activates cell-mediated immune responses, such as proliferation and activation of type I helper T (Th1) cells. Given that cytotoxic T-cells and suppressor T-cells were activated to proliferate in this study, *Propolis* administration stimulated the proliferation of Th1 cells via the increase of IFN-γ and the activation of the cell-mediated immune response of cytotoxic T-cells (Fukuhara *et al.*, 2002; Duarte *et al.*, 2003). Evidently, the reduction of antibody production observed in previous studies was caused by the stimulation of the cell-mediated immune response due to an increase in IFN-γ and the suppression of the humoral immune response. Since suppressor T-cells inhibit antibody production, the increase in suppressor T-cells may decrease antibody production. Unfortunately, it was not confirmed whether cytotoxic T-cells or suppressor T-cells were increased. Therefore, it is necessary to clarify further the T-lymphocyte subsets involved and the cytokine production profiles more thoroughly in order to elucidate the effect of *Propolis* on the immune system. CD8-positive cells decreased in the *Propolis* with irradiation group more than in the irradiation alone group. It may be possible to render this study more reliable by repeated experiments.

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