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Red Ginseng Marc and Steamed Extraction Powder Enhance Proliferation and Inflammatory Cytokine Modulation in Canine PBMCs Stimulated by IL-2

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Abstract The growing market for companion animals, combined with their increasing lifespan, has generated an increased interest in companion animal immunity enhancers. Ginsenoside, a saponin component of ginseng and an essential ingredient of red ginseng marc (produced during red ginseng production), is effective in improving immunity. In this experiment, a powder mixture of red ginseng marc and steamed red ginseng extract powder (RGME) was orally administered to dogs for eight weeks. Subsequently, blood samples were collected and tested every four weeks. In addition, canine peripheral blood mononuclear cells (cPBMCs) were stimulated with or without interleukin-2 (IL-2) to evaluate their proliferation and cytokine secretion abilities. Proliferation assay suggests that the administration of RGME effectively enhanced numbers of cPBMCs under IL-2 stimulation. Furthermore, in the RGME group, a significant increase in the concentration of interferon gamma released from cPBMCs under IL-2 stimulation was observed. In conclusion, RGME might be an effective health supplement for improving immunity in dogs.

Key words dog, immunomodulation, peripheral blood mononuclear cell, red ginseng marc, steamed red ginseng extract.

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Introduction

The growing population of companion animals has resulted in rapid growth in the companion animal health market. Due to the increased life expectancy of companion animals, the proportion of animals with immune and geriatric disease has also grown, and there is now a focus on products that will enhance immunity in companion animals.

Ginseng, a well-known oriental medicinal herb, is a known immunomodulator. Red ginseng, also referred to as dried brown ginseng, is the product that remains after steaming ginseng without removing the skin. The main pharmacologically active ingredient of ginseng is a saponin called ginsenoside. Ginsenoside modulates body function control by affecting the central nervous system, endocrine system, immune system, and metabolic system (16,17,25). The ginsenoside changes when red ginseng is manufactured (12). Although red ginseng has a less complete pharmacology than ginseng, its immunity enhancement and infection prevention properties are superior to ginseng in its original form (15). As a result of growing awareness of the effects of red ginseng, due to clinical research, the production and consumption of red ginseng and red ginseng marc (a byproduct obtained when processing ginseng extract, through heating with water or alcohol) has increased. Red ginseng marc is currently produced at a rate of approximately 700 tons per year. However, there are still many additional ginsenoside components in red ginseng, and it may be beneficial to further investigate the utilization of these components.

In vivo studies elucidating the physiological activity of red ginseng, have revealed that the administration of red ginseng extract is effective in improving the postoperative nutritional status and immune function of the gastric and digestive system in cancer patients (13). Its benefits also include the lowering of cholesterol (21), inhibition of platelet aggregation (20), antioxidative activity (22), and inhibition of skin cancer progression (19). Studies on the application of ginseng and red ginseng by-products are also being conducted within the livestock industry. For example, studies evaluating the effect of red ginseng marc on the productivity of poultry and on the quality of pork have reported positive benefits (2,7,8,24). As a result, feed additives made from red ginseng marc are being made for industrial livestock to enhance their immunity. Furthermore, red ginseng has been proven effective for race horses and puppies (5,10). This has resulted in the production of food using red ginseng roots and ginseng byproducts for these animals (4). However, there is a lack of evidence on the effect of red ginseng marc on the immunity of canine companion animals. The goal of this study was to evaluate whether red ginseng marc combined with steamed red ginseng extract powder (RGME) could enhance the proliferation and secretion of inflammatory cytokine modulation in canine peripheral blood mononuclear cells (PBMCs) stimulated by interleukin-2 (IL-2).

Materials and Methods

Animal experiments

Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) (SNU-160819-14; Seoul National University, Seoul, Korea), and conducted in compliance with regulations. Beagle dogs (n = 8, age: 1-3 years; weight: 7-10 kg) were obtained from Raon Bio Animal, Inc. (Yongin, Korea). All dogs were free from canine distemper; parvo and corona virus infections; and showed normal blood analysis (complete blood count; CBC and serum biochemistry) and imaging findings (X-ray and abdominal ultrasonography). The dogs were caged in a room maintained at a constant temperature of 25°C with a humidity level of 50%, and a 12-hour light/dark cycle. They were randomly divided into two different groups, i.e., control (n = 3) and experimental (n = 5) groups. Each group received the same feed twice a day, and only one group received 10 g of the RGME (containing 8 g of 4% red ginseng marc powder, and 2 g of 1% steamed red ginseng extract powder; provided by the Korea Ginseng Cooperation, Daejoen, Korea) mixed with water once daily. Blood samples were collected every 4 weeks. At the end of experiment, none of the animals were euthanized but were either adopted into a family home or used in later experiments.

Canine peripheral blood mononuclear cell (cPBMC) isolation

Canine blood samples were obtained from the jugular or cephalic vein with a 24-gauge needle and diluted with an equal volume of phosphate buffered saline (PBS). Next, cPBMCs were isolated from the blood samples using a density gradient medium (Ficoll-Paque Plus, GE Healthcare, Uppsala, Sweden). First, 15 mL of Ficoll-Paque Plus was added to the bottom of a 50-mL conical tube. Following this, 10 mL of 1:1 mixture of whole canine blood and PBS was added and spun at 400 × g for 30 min, with acceleration set to 1 and deceleration set to 0. The resulting mononuclear layer was collected and washed twice with PBS. cPBMCs were resuspended in the Roswell Park Memorial Institute (RPMI) 1640 medium (PAN Biotech, Aidenbach, Germany), containing 20% fetal bovine serum (FBS; PAN Biotech).

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cPBMC proliferation assay and proliferation ratio calculation

cPBMCs were obtained from both the experimental and control groups, every 4 weeks. cPBMCs (1×10^5 cells/well) were seeded in 96-well plates (SPL Life science, Pocheon, Korea) and cultured in the RPMI-1640 medium (200 μL/well) containing 20% FBS with or without canine recombinant interleukin-2 (crIL-2; 10 ng/mL, R&D systems, Minneapolis, MN, USA), at 37°C in a humidified 5% CO_2 incubator. Every 2-3 days, for one week, 100 μL of the supernatant medium was carefully removed with a pipette, without disturbing the lower layer, and immediately added to 100 μL of fresh RPMI-1640 medium containing 20% FBS with or without crIL-2 (10 ng/mL). The proliferation of cPBMCs from each group was measured by cell viability assay using a D-Plus™ CCK Cell Kit (Dongin LS, Seoul, Korea), according to the manufacturer's instructions. In brief, the CCK reagent (10 μL) was added to each well and incubated for 1 hour. Then, the absorbance values at 450 nm were measured using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). In addition, proliferation ratio was calculated as the CCK absorbance profile of cPBMCs treated with crIL-2 divided by that of cPBMCs treated without crIL-2.

Cytokine assay

Isolated cPBMCs (1×10^5 cells/well) were cultured in 200 μL of RPMI-1640 medium containing 20% FBS, in 96-well plates, and treated with or without crIL-2 (10 ng/mL) for 24 hours in a humidified 5% CO_2 incubator at 37°C. Next, the supernatant was harvested and centrifuged to remove any impurities, and the centrifuged supernatants were stored at – 80°C.

Enzyme-linked immunosorbent assay (ELISA) and cytokine ratio calculation

Cell-free supernatants were analyzed to assess immunological activity. Canine tumor necrosis factor-alpha (TNF-α) Quantikine ELISA Kit, canine interleukin-1 beta (IL-1β) DuoSet ELISA Kit, and canine interferon gamma (IFN-γ) Quantikine ELISA Kit (all from R&D systems) were used according to recommended instructions. In addition, cytokine ratio was calculated as the concentration of each cytokine from cPBMCs treated with crIL-2 divided by that from cPBMCs treated without crIL-2.

Hematology and serum biochemistry

During the treatment period, all animals were observed for clinical signs and fasted for 12 hours before blood sampling. Blood samples were obtained from the jugular or cephalic vein of the dogs with a 24-gauge needle. White blood cells (WBC), red blood cells (RBC), hematocrits (HCT), and platelets (PLT) were measured using a hematological autoanalyzer (ADVIA 2120i, Siemens, Erlangen, Germany). Serum biochemical examination was performed for each animal for which a hematology examination had been conducted. Serum samples were obtained after centrifugation (3,000 rpm, 5 min) of the blood samples and analyzed using a clinical chemistry autoanalyzer (Hitachi 7180, Hitachi, Japan) to determine the levels of aspartate aminotransferase (AST); alanine aminotransferase (ALT); alkaline phosphatase (ALP); total bilirubin (TBIL); gamma-glutamyl transferase (GGT); blood urine nitrogen (BUN); creatinine (CREA); calcium (Ca); inorganic phosphorus (IP); glucose (GLU); total protein (TP); albumin (ALB); chloride (Cl); sodium (Na); and potassium (K).

Statistical analysis

Data are shown as mean ± standard deviation. Group means were compared using one-way analysis of variance (ANOVA) using the GraphPad Prism software (version 6.01, GraphPad Inc., La Jolla, CA, USA). A p-value of < 0.05 was considered statistically significant.

Table 1. Hematological values for control and experimental groups

Parameter	Control group				Experimental group		
	References	Day 0	4 week	8 week	Day 0	4 week	8 week
Hematological values							
WBC ($\times 10^3/\mu L$)	(5.2-17)	9.01 ± 0.64	8.27 ± 0.98	7.53 ± 0.6	7.68 ± 1.85	7.71 ± 1.04	7.09 ± 0.33
RBC ($\times 10^6/\mu L$)	(3.71-5.70)	7.87 ± 0.17	8.05 ± 0.4	7.92 ± 0.36	7.5 ± 0.41	7.44 ± 0.55	7.73 ± 0.72
HCT (%)	(14.3-40.0)	53.53 ± 1.06	54.8 ± 2.14	51.7 ± 2.92	52.74 ± 3.07	50.88 ± 4.31	53.24 ± 5.47
PLT ($\times 10^4/\mu L$)	(14.0-40.0)	25.1 ± 3.39	23.8 ± 2.32	20.7 ± 1.71	25.8 ± 2.98	26.72 ± 3.74	25.2 ± 6.4

Values are mean ± standard deviation.

Control group (no supplement RGME), n = 3; Experimental group (supplemented with RGME), n = 5.

Table 2. Biochemical serum values for control and experimental groups

Parameter	Control group				Experimental group		
	References	Day 0	4 week	8 week	Day 0	4 week	8 week
AST (IU/L)	(10-43)	48.33 ± 18.11	29 ± 4.24	26 ± 3.26	26.6 ± 6.28	24.4 ± 7.47	25.4 ± 6.8
ALP (IU/L)	(0-97.9)	29 ± 9.93	26.33 ± 10.65	32 ± 10.61	50.2 ± 24.71	67 ± 39.54	59.8 ± 35.16
BUN (mg/dL)	(9.6-31.4)	18.7 ± 8.91	15.26 ± 4.29	12.93 ± 1.97	10.9 ± 1.84	11.04 ± 3.31	13.12 ± 2.26
CREA (mg/dL)	(0.4-1.3)	0.73 ± 0.1	0.64 ± 0.07	0.75 ± 0.08	0.58 ± 0.04	0.59 ± 0.04	0.6 ± 0.04
GLU (mg/dL)	(74.5-120)	70.66 ± 7.13	98 ± 1.41	107.33 ± 6.12	76.8 ± 11.17	90 ± 10.48	100.8 ± 9.26
TBIL (mg/dL)	(0-0.2)	0.03 ± 0.01	0.03 ± 0.04	0.04 ± 0.04	0.06 ± 0.01	0.03 ± 0.02	0.01 ± 0.01
ALB (g/dL)	(2.6-4.4)	3.98 ± 0.25	4.15 ± 0.2	4 ± 0.18	4.06 ± 0.25	3.92 ± 0.25	3.99 ± 0.2
TP (g/dL)	(5.7-7.5)	7.65 ± 0.13	7.35 ± 0.42	7.3 ± 0.26	7.32 ± 0.16	7.03 ± 0.26	7.29 ± 0.11
GGT (IU/L)	(0-14)	1.33 ± 0.94	2.66 ± 1.88	6.66 ± 1.24	2.4 ± 2.49	2.6 ± 1.49	6 ± 1.78
CA (mg/dL)	(9.0-11.9)	11.96 ± 0.83	11.86 ± 0.75	12.5 ± 1	11.54 ± 0.1	11.3 ± 0.3	11.8 ± 0.26
IP (mg/dL)	(1.3-6.3)	5.73 ± 0.81	5.33 ± 0.47	5.36 ± 0.82	4.84 ± 0.28	5.32 ± 0.66	5.56 ± 0.51
ALT (IU/L)	(5.8-83.3)	36.33 ± 7.84	49 ± 4.96	47.33 ± 2.05	54.6 ± 14.4	54.6 ± 15.53	59 ± 17.62
Na (mmol/L)	(145.1-152.6)	146.43 ± 0.37	146.5 ± 0.37	150.1 ± 0.85	147.46 ± 1.23	148.46 ± 1.08	155.12 ± 2.78
K (mmol/L)	(3.6-5.5)	4.83 ± 0.09	4.56 ± 0.28	5.01 ± 0.59	4.62 ± 0.44	4.54 ± 0.43	4.79 ± 0.22
Cl (mmol/L)	(113.2-122.9)	112.63 ± 1.14	115.2 ± 0.74	119.26 ± 0.44	115.04 ± 2.15	116.92 ± 0.8	124.84 ± 2.17

Values are mean ± standard deviation.

Control group (no supplement RGME), n = 3; Experimental group (supplemented with RGME), n = 5.

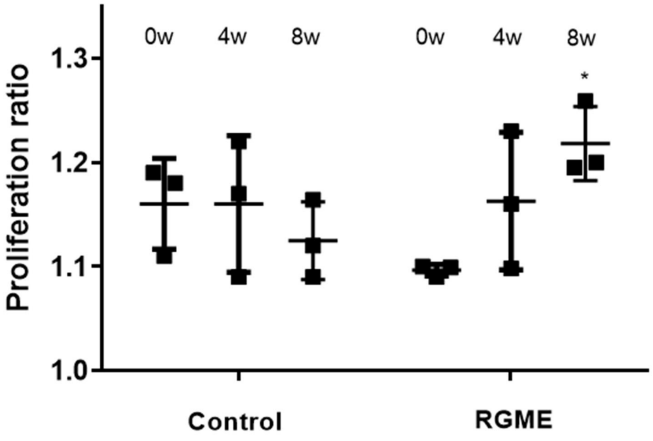

Fig. 1. Enhanced proliferative capacity of crIL-2 stimulated PBMCs derived from dogs fed red ginseng marc and steamed red ginseng extract powder (RGME). PBMCs collected from dogs fed RGME showed enhanced proliferative ability after 8 weeks. Data are shown as mean ± standard deviation of three independent experiments. *Compared with 0-week in RGME group, p-value < 0.05.

Results

Clinical signs and blood analysis

During the study, no clinical signs of distress (including vomiting, anorexia, and diarrhea) were observed in animals treated with the RGME mixture (10 g/day) for 8 weeks. Results from the hematological examination demonstrated that complete blood count of the experimental group was normal, and was not significantly different from the control group. Serum biochemistry results also showed no significant differences between the control and experimental group (Tables 1, 2).

Evaluating the proliferation ratio of cPBMCs

To assess the immune-enhancing effects of RGME, lymphocyte proliferation assay was performed using cPBMCs (stimulated with or without crIL-2) from control and RGME groups across multiple time-points. Comparison of proliferative ratio revealed that there were no significant differences at 0-, and 4-week in both groups. However, in RGME group, the proliferation ratio at 8-week was significantly higher than that at 0-week (Fig. 1).

Evaluating the cytokine ratio of cPBMCs

The concentration of pro-inflammatory cytokines (TNF-α, IL-1β, and IFN-γ) released by cPBMCs stimulated with or without crIL-2 were used to calculating cytokine ratio to evaluate whether immune cells collected from dogs fed RGME could show enhanced cytokine-producing capacities. There were no significant differences in TNF-α and IL-1β concentration from cPBMCs derived from control and RGME dogs. However, significant difference was found in IFN-γ concentration from cPBMCs derived from RGME dogs at 4-, and 8-week (Fig. 2).

Discussion

In the present study, we found that RGME, containing 8 g of 4% Red ginseng marc powder and 2 g of 1% steamed red

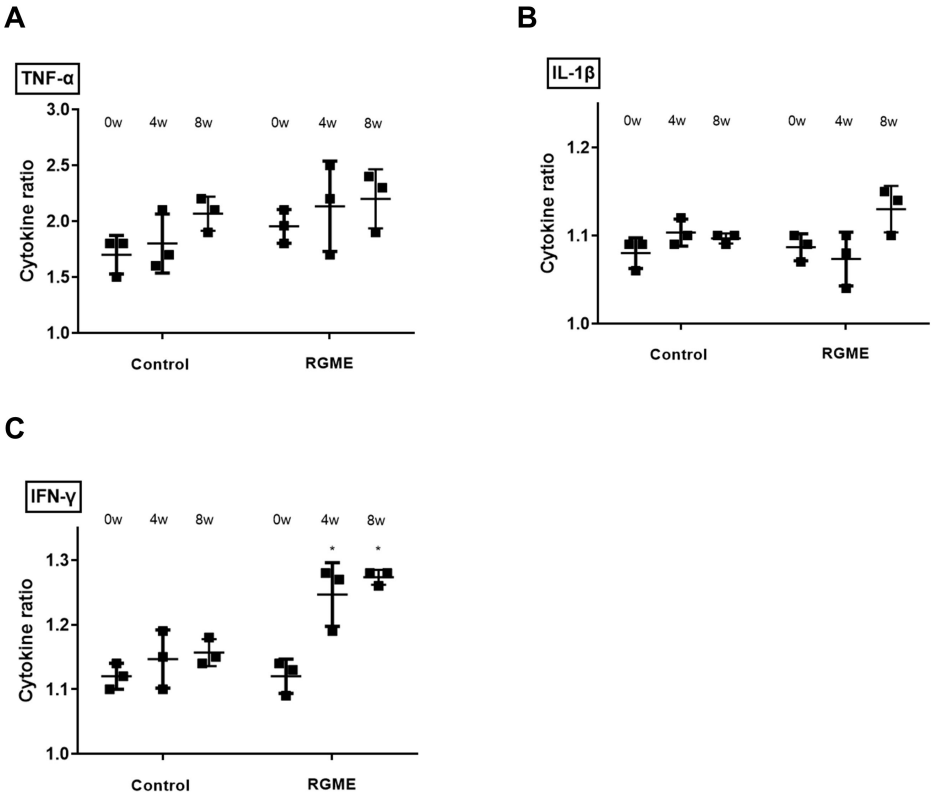

Fig. 2. Changes in secretion capacity of pro-inflammatory cytokines in cPBMCs derived from dogs fed red ginseng marc and steamed red ginseng extract powder (RGME). There were no significant differences in TNF-α and IL-1β concentrations between control and RGME groups. However, IFN-γ concentrations of cPBMCs derived from the RGME group at 4-, and 8-week were significantly increased compared to those at 0-week. Data are shown as mean ± standard deviation of three independent experiments. *Compared with 0-week in RGME group, p-value < 0.05.

ginseng extract powder, showed immunomodulatory effects in canine PBMCs. Although we could not measure exact concentration of active ginsenoside in RGME in this study, following examination of the results from assays conducted using cPBMCs, we concluded that RGME was safe and effective for enhancing immunity. Randomized double-blind comparisons between the experimental and control groups, after the ingestion of RGME, confirmed the immunity enhancement.

Compared to other herbal medicines, red ginseng is known to have few side effects, but blood pressure elevation, tachycardia, appetite reduction, behavioral changes, and non-inflammatory hepatitis may occur when first ingested (18). Therefore, physical examination and abnormal behavior were observed for two months after the first oral administration of red ginseng powder, and general blood tests were performed every four weeks. In the RGME group, no dogs showed clinical symptoms of distress, and their feed intake was consistent. Our CBC analysis yielded no significant differences between the RGME and the control group; and serum chemistry showed that the levels of AST, ALT, GGT, and ALP (markers for liver function) remained stable and normal. In addition, the levels of BUN, CREA, CA, and IP (markers for renal function) were normal, and no abnormalities in the levels of blood glucose, blood protein, and electrolytes were found. These findings further confirm that ginseng powder is safe to use and does not adversely affect body functions.

IL-2, known as T-cell growth factor, induces the proliferation and activity of T cells. This is due to the IL-2 receptor in PBMC responding to IL-2. The degree of proliferation can be an indicator of immunity levels (9,11,23). In the present study, the proliferation ability of IL-2 stimulated cPBMC was evaluated. Comparing the RGME group with the control, the degree of cPBMC proliferation was statistically insignificant at four weeks, but significantly increased at eight weeks. Taken together, these results suggest that the immune cells of dogs fed RGME proliferate rapidly when they are stimulated by external stimuli.

Cytokines are proteins secreted by immune cells, and are important mediators of the immune system, crucial in identifying the causes and treatments for specific diseases as well as immunological and immunosuppressive factors. Therefore, we investigated the effects of RGME on dogs by measuring the levels of pro-inflammatory cytokines such as TNF-α, IL-1β, and IFN-γ secreted by cPBMC stimulated with IL-2 (3,14). IFN-γ is mainly secreted by T cells and natural killer cells (NK) and activates macrophages, enhancing humoral immunity to antigens (1,6). Moreover, IFN-γ production has been reported

to be a marker of cellular immunity to some infections. In this study, IFN-γ concentration was significantly elevated in the RGME group at 4-, and 8-week. Taken together, these results suggest that the immune cells of dogs fed RGME release more pro-inflammatory cytokines when they are stimulated by external stimuli.

In conclusion, our study indicates that the immune cells of dogs fed RGME produce a larger immune response than those of the control group, in the presence of external stimuli. In other words, feeding RGME to dogs might increase their cellular immunity.

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Conflicts of Interest

The authors have no conflicting interests.

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