The Role of Supplementary Dietary Antioxidants on Immune Response in Puppies*

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CLINICAL RELEVANCE

This study evaluated the effect of supplementary antioxidants and whey protein on immune system challenges in young animals. Four groups of 10 puppies each were fed a control diet (CTRL) or one of three test foods (control + antioxidants [AOX], control + antioxidants + 1% whey protein [WPI], or a grocery-store brand diet [GROC]) for 6 weeks. A standard vaccination protocol with a combination canine parvovirus (CPV) and distemper (CDV) vaccine was carried out at 2 and 4 weeks. The results showed that animals on high AOX foods had significantly increased response to the CDV vaccine and increased CD4+/CD45+ dim cells, indicative of memory cells, compared with the CTRL group and increased serum vitamin E concentrations compared with the CTRL, WPI, and GROC groups.

INTRODUCTION

The antioxidant defense system in the body is complex and involves enzymes, proteins, vitamins, and other molecules. It works together with exogenous antioxidants from sources such as food to protect cells from free radicals generated during an activated immune response and inflammation. During periods of immune challenge (e.g., vaccinations), immune cells generate free radicals (e.g., superoxide, hydrogen peroxide, hydroxyl radicals), which have the potential to damage cells and tissue in the body. The extent of the damage depends on the type or nature of free radicals, their site of generation, and the antioxidant status of the body. The body’s antioxidant defense system,
along with exogenous antioxidants in food, helps to reduce oxidative stress and protect cells from harmful free radicals. Antioxidants such as vitamins E and C, β-carotene, and selenium may function in this way to prevent excessive inflammation. Deficiency of one or more of the antioxidants can cause clinical signs to manifest in a short time, as shown in growing dogs deficient in vitamin E and selenium. A survey

The developing immune system in young dogs may benefit from antioxidant supplementation.

study that looked at three groups of puppies commercially obtained for research purposes showed moderate to severe deficiencies of vitamin E in the animals as a result of their stressful environments, low intake of vitamin E, or both. These deficiencies resulted in severe clinical syndromes of hemoglobinuria, lipofuscinosis, and segmental retinal degeneration.

Although the Association of American Feed Control Officials (AAFCO) has recommended a minimum vitamin E intake in dogs of 50 IU/kg, most of the immune function studies have tested levels five to eight times higher. Supplementation at concentrations higher than those required for reproduction and growth may benefit an animal’s immune function by providing protection against potential prooxidants in food, such as nitrates and iron. Dietary supplementation with α-tocopheryl acetate is able to stimulate CD8+ T cell population in geriatric dogs regardless of the ratio of omega-6 (n-6):omega-3 (n-3) fatty acid intake. Because of their age, the geriatric population is considered immunocompromised, and supplementation with vitamin E may be beneficial for immune function. Similarly, at the other end of the age spectrum, young dogs face challenges from immunization, and their developing immune system may benefit from antioxidant supplementation.

Previous studies have demonstrated a beneficial effect associated with administration of a single antioxidant during an immune response. Antioxidant supplementation in dogs, as evidenced by increased serum levels of vitamin E, resulted in higher rabies-specific neutralizing antibodies at 2, 4, and 6 weeks after vaccina-
several studies looking at the effect of antioxidants on free radical production or aging, it is clear that a combination of antioxidant molecules is more effective than a single source.\textsuperscript{16,17} A possible reason for the improved efficacy of antioxidant combinations is that different antioxidants function better in different environments: Some work in the aqueous phase and others in the lipid phase. Vitamin E performs better in environments with high oxygen tension, whereas β-carotene is best at low oxygen tension. Thus, some antioxidants are more effective than others in organs such as the lungs.\textsuperscript{18} Vitamin C is a water-soluble antioxidant that works primarily in the extracellular phase, while vitamin E is a lipid-soluble antioxidant that is effective in the cellular and membrane environments. Vitamin C is also involved in the regeneration of vitamin E in the body. Selenium, on the other hand, is a constituent of the enzyme glutathione peroxidase, which generates the endogenous antioxidant glutathione.

Milk contains antioxidative and nutritive factors, such as lactoferrin, enzymes, immunoglobulins (IgA and IgM), and nucleotides, which have been shown to stimulate the immune system as well as maintain gastrointestinal tract health.\textsuperscript{19} Other smaller peptides and oligosaccharides in whey, such as lactoperoxidase, glycomacropeptide, phospholipids, β-lactoglobulin, lactoferricin, and the digestion products of these compounds, are also able to contribute to the beneficial effects of milk proteins, such as preventing bacterial infection and regulating the immune system.\textsuperscript{20,21}

Vaccination is a method of deliberately stimulating the adaptive immune system to “prime” it for potential infections by generating antigen-specific effector cells. A successful vaccine for viruses should elicit an inflammatory response, particularly CD8+ T lymphocytes, but at the same time should generate neutralizing antibodies. The vaccine should also generate long-lived immunologic memory (priming of B and T cells).\textsuperscript{22} Memory T cells are defined as lymphocytes that mediate immunologic memory; they are more sensitive to antigen than naïve lymphocytes and are able to respond more rapidly when reexposed to the antigen that originally induced them.\textsuperscript{22} Memory T cells can be distinguished from naïve T cells via differences in expression of CD45RA.\textsuperscript{23} The standard vaccination protocol for puppies with a distemper (CDV) and parvovirus (CPV) combination modified-live virus vaccine (V7) was used to stimulate the immune function and determine the effect of antioxidant supplementation of food in puppies. Previous studies in which antioxidants such as vitamins E and C and β-carotene were fed alone or in various combinations to dogs and chickens demonstrated improved immune response to vaccinations.\textsuperscript{24,25}

The effectiveness of a high-antioxidant food on immune response was assessed.

In our study, the effectiveness of a high-antioxidant food on immune response was assessed through vaccination titers, lymphocyte proliferation against specific viral antigens and nonspecific mitogens, natural killer cell activity, and generation of memory T cells. These are measures that indicate development of antibodies, inflammatory response to infection, and generation of effective and long-term immunologic memory.
MATERIALS AND METHODS

Healthy male and female puppies (N = 40) with no prior infections were chosen for the study. Puppies are known to be prone to diarrhea related to their immature intestinal tract, and thus signs of diarrhea, if present, were treated according to standard veterinary practice before the study began. Four groups of 10 puppies each were fed a control diet (CTRL) or one of three test foods (control + antioxidants [AOX], control + antioxidants + 1% whey protein [WPI], or a grocery-store brand with low antioxidants [GROC]) for 6 weeks. All foods were complete and balanced for puppies, with vitamin E levels exceeding the AAFCO recommendation of 50 IU/kg. The AOX combination consisted of vitamin E (500 IU/kg dry-matter basis [DM]), vitamin C (70 mg/kg DM), β-carotene (0.4 mg/kg DM), and selenium (0.8 mg/kg DM). Dietary analyses of the diets are shown in Table 1. The protocol was approved by Hills’ Pet Nutrition IACUC committee in compliance with the guidelines set by Hill’s Pet Nutrition on animal care and use.

### Treatment Groups

A total of 40 puppies, 7 weeks of age, were selected for the 6-week study, with 10 puppies in each dietary group. Meals were fed twice daily (morning and afternoon) for a 1-hour period. Body weight was measured on days 0, 7, 14, 21, 28, 35, and 42. Food intake data were collected daily. Exclusion criteria included loss of 2% of body weight or refusal to eat for more than 2 days, at which point puppies were to be removed from the study; however, no puppies were excluded. On days 1 and 42, blood was collected for complete blood counts, serum chemistry profiles, and determination of serum levels of vitamins E and C. On days 1 and 34, blood was collected for lymphocyte proliferation and lymphocyte subset analysis.

### TABLE 1. Analysis of the Nutrient Contents of the Different Foods

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CTRL</th>
<th>AOX</th>
<th>WPI</th>
<th>GROC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (%)</td>
<td>6.1</td>
<td>6.8</td>
<td>6.8</td>
<td>7.9</td>
</tr>
<tr>
<td>Protein (Kjeldahl method; %)</td>
<td>28.6</td>
<td>29.2</td>
<td>29.3</td>
<td>26.9</td>
</tr>
<tr>
<td>Crude fiber (%)</td>
<td>3.8</td>
<td>2.8</td>
<td>2.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>7.4</td>
<td>8.1</td>
<td>7.7</td>
<td>5.8</td>
</tr>
<tr>
<td>Crude fat (%)</td>
<td>17.8</td>
<td>16.5</td>
<td>16.4</td>
<td>14.0</td>
</tr>
<tr>
<td>Insoluble fiber (%)</td>
<td>7.4</td>
<td>9.0</td>
<td>17.6</td>
<td>5.7</td>
</tr>
<tr>
<td>Soluble fiber (%)</td>
<td>1.5</td>
<td>2.6</td>
<td>9.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Δ-Tocopherol (µg/g)</td>
<td>3.4</td>
<td>2.6</td>
<td>3.5</td>
<td>3.0</td>
</tr>
<tr>
<td>γ-Tocopherol (µg/g)</td>
<td>15.1</td>
<td>12.0</td>
<td>16.0</td>
<td>7.7</td>
</tr>
<tr>
<td>α-Tocopherol (µg/g)</td>
<td>4.0</td>
<td>3.8</td>
<td>5.3</td>
<td>1.3</td>
</tr>
<tr>
<td>α-Tocopherol acetate (µg/g)</td>
<td>126.4</td>
<td>454.3</td>
<td>581.4</td>
<td>67.0</td>
</tr>
<tr>
<td>Total tocopherols (µg/g)</td>
<td>133.1</td>
<td>460.4</td>
<td>589.6</td>
<td>70.0</td>
</tr>
<tr>
<td>β-Carotene (µg/g)</td>
<td>0.1</td>
<td>0.3</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>Ascorbic acid (µg/g)</td>
<td>6.2</td>
<td>66.7</td>
<td>64.7</td>
<td>nd</td>
</tr>
</tbody>
</table>

AOX = antioxidant diet; CTRL = control diet; GROC = grocery brand diet; nd = not detected; WPI = whey protein diet.
Vaccination Protocol

The puppies’ standard vaccination protocol was administered during the study. Puppies had not received any prior vaccinations. The combination CDV and CPV modified-live virus vaccine (Progard-7, Intervet) was administered on day 14, and a booster was given on day 28. Blood samples were obtained weekly, and serum was isolated and sent for measurement of titers from day 14 to day 42. The titers were measured at the Cornell University School of Veterinary Medicine Diagnostic Laboratory using the CPV-2 hemagglutinin inhibition test and the CDV serum neutralization test.

Lymphocyte Isolation

Lymphocytes were isolated by density gradient centrifugation (30 minutes at 500 \( \times \) g) of diluted blood (one part blood:one part HBSS [Hanks balanced salt solution] + 25 mM HEPES, both from Life Technologies, Grand Rapids, MI) layered over Accu-paque (Accurate Chemical, Westbury, NY). Mononuclear cells were collected from the interface and washed twice with HBSS and once with AIM-V media with 2 mM l-glutamine, 25 mM HEPES, and 50 \( \mu \)g/ml gentamicin (Life Technologies). For enumeration of leukocytes, a sample of the cells (40 \( \mu l \)) was diluted in 10 ml of Nova Celltrak Isotonic Diluent, and any remaining erythrocytes were removed by the addition of Zap-oglobin II Lytic Reagent (both from Beckman-Coulter, Miami, FL). Cells were counted with Nova Celltrak II twice for an average of two values. Cells were diluted to 2 \( \times \) 10\(^6\) cells/ml in complete medium (RPMI-1640 [Life Technologies] supplemented with 2 mM l-glutamine, 25 mM HEPES, 50 \( \mu \)g/ml gentamicin, and 10% fetal bovine serum [JRH Biosciences, Lenexa, KS]) for natural killer cell assays, mitogen-stimulated proliferation assays, and mitogen-stimulated lymphocytes for real-time polymerase chain reaction and in supplemented AIM-V medium for proliferative assays to specific antigen (vaccine components).

Lymphocyte Proliferation

Proliferation of lymphocytes was measured by [3H]thymidine incorporation into DNA, after mitogen or specific antigen stimulation. Lymphocytes were plated at a concentration of 1 \( \times \) 10\(^6\) cells/ml in Costar 96-well low-evaporation plates (Corning, Acton, MA). The plates were incubated for 91 hours (specific antigen) or 54 hours (mitogen) at 37\(^\circ\)C, 100% humidity, and 7% carbon dioxide. Plates were pulsed with 1 \( \mu Ci/well \) of [3H]thymidine and incubated for 18 hours at 37\(^\circ\)C, 100% humidity, and 7% carbon dioxide. Cellular DNA was harvested onto glass-fiber filters using a Skatron Combi Cell Harvester (Skatron Instruments, Norway). Corresponding samples of glass-fiber paper were suspended in CytoScint ES (ICN, Irvine, CA). [3H]thymidine uptake was counted using Packard TriCarb 2100TR Liquid Scintillation Analyzer (Hewlett-Packard, Downer's Grove, IL).

The lymphocytes were artificially stimulated using the following cytokines:

- Phytohemagglutinin (PHA; Wellcome Diagnostics, Dartford, UK), 2 \( \mu \)g/ml (T cell activation)
Concanavalin A (ConA; Sigma-Aldrich, St. Louis, MO), 5 µg/ml (T cell activation)

Pokeweed mitogen (PWM; Sigma-Aldrich), 20 µg/ml (T and B cell activation)

Lipopolysaccharide (LPS; Sigma-Aldrich), 20 µg/ml (B cell activation)

T cells were also stimulated using a parvovirus modified-live virus vaccine (Progard-CPv, Intervet) and a canine distemper–adenovirus type-2–parainfluenza–parvovirus modified-live virus vaccine (Progard-7, Intervet). Progard-7 was used independently in two separated portions for specific-antigen stimulation: a *Leptospira canicola*–*icterohaemorrhagiae* bacteria portion and a canine distemper–adenovirus type-2–parainfluenza–parvovirus vaccine portion (designated as V7). In addition, individual CPV particles were used in separate tests of specific antigen stimulation.

**Flow Cytometric Analysis**

To examine the proportion of CD4 and CD8 cells in comparison with a naïve/memory cell marker, CD45RA, aliquots of whole blood were co-stained with canine specific antibodies: αCD4-FITC (rat clone YKIX302.9), αCD8a-phycoerythrin (PE; rat clone YCATE55.9), and αCD45RA (mouse clone CA4.1D3) (all from Serotec, Raleigh, NC). Staining for CD45RA was performed first as a three-step protocol. After incubation with a saturating concentration of αCD45RA, erythrocytes were lysed with ammonium chloride solution, washed twice with phosphate buffered saline (PBS)/0.1% sodium azide (NaAzide), and incubated with a second step of goat anti-mouse IgG1 biotin conjugate (BD-PharMingen, San Jose, CA). After washing twice with PBS/0.1% NaAzide, cells were stained with a third step of strep-avidin cychrome (BD-PharMingen) and simultaneously stained with αCD4-fluorescein-5-isothiocyanate (FITC) and αCD8a-PE. Two isotype control tubes were stained in parallel for each sample containing αCD45 along with isotype controls for FITC and PE as well as an isotype for the mIgG1 + anti-IgG1-biotin + strep-avidin cychrome. Cell staining was measured at the Iowa State University Cell and Hybridoma Facility using an Epics XL-MCL (Beckman-Coulter). Although previous reports of this antibody demonstrated both positively and negatively stained populations of CD45 cells, our use of a biotinylated second-step antibody in conjunction with strep-avidin-cychrome gave 99.7% positively stained cells. CD4+ and CD8+ cells both demonstrated two distinct peaks. The CD4/CD45 bright and CD8/CD45 bright are considered naïve cells, and the CD4/CD45 dim and CD8/CD45 dim are considered memory cells.

**Natural Killer Cell Activity**

Natural killer cell activity was measured using a 51Cr release assay using canine thyroid adenocarcinoma (CTAC) cells. An increase in cytotoxicity would increase release of 51Cr from the target cells. Target cell lines (CTAC) were incubated with radiolabelled Cr and effector cells (peripheral blood mononuclear cells, containing natural killer cells) at effector:target ratios of 40:1 and 80:1. In parallel cultures of effector:target, recombinant human interleukin-2 (IL-2; Sigma) was added to the mixture. IL-2 stimulates the activity of the natural killer cell via its intermediate affinity IL-2 receptor. The cells were obtained 4 days after the booster vaccination. For the 40:1 effector:target wells, target cells were added for final concentration of 5 × 10⁴ cells/well and effector cells for a final concentration of 2 × 10⁵ cells/well. A final volume of 270 µl was made up with complete medium. Cultures were in-

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Cunnick J: Personal communication, Department of Animal Science, School of Veterinary Medicine, Iowa State University, Ames IA.
cubated for 16 hours at 37°C and 7% carbon dioxide in a humidified incubator. For wells with IL-2, 1 ng/well was added. For maximum release wells, 10% trichloroacetic acid was added to targets at the end of the assay to a volume of 270 µl. Plates were centrifuged at 50 \times g at room temperature for 5 minutes. Supernatant (120 µl) was collected and transferred to respective counting tubes, and 51Cr release was counted using the Gamma Trac Automatic Gamma Counting System (TM Analytic, Elk Grove Village, IL).

Statistical Analysis
Analysis of variance (covariance) was performed using the GLM procedure of the SAS system with treatment as the independent variable. Titers were analyzed using split-plot analysis and multivariate analysis of repeated measures.

RESULTS

Body Weights and Food Intake
There were no significant differences in weight gain between any of the groups on the different foods (Figure 1). Weight gains in the 6-week period ranged from 3.1 to 3.5 kg. Food intake also was not different among the groups, with average intake ranging from 205 to 226 g/day.

Vaccination Titers
The antibody response to the CDV vaccine was measured by quantifying the serum-neutralizing antibodies. The antibody titer increased in all the groups after vaccination but was significantly higher in the group on the AOX food compared with all the other groups (P < .05; Figure 2). The response was also significantly higher at week 4 for the AOX group compared with the CTRL group (P < .05) and tended to be higher than the GROC group (P = .07). The response to the CPV vaccine was significantly higher for the AOX group at weeks 2 and 3 compared with the CTRL and WPI groups (P < .05; Figure 3).

Lymphocyte Subpopulation and Proliferation
The percent of memory CD4+ cells, as defined by the CD4/CD45 dim cells, was significantly higher in the AOX group compared with the CTRL group after vaccination (Table 2). All the memory CD8 cells increased after vaccination, but the differences among the groups were not significant.

Stimulation with the V7 combination antigen resulted in a significantly higher lymphocyte proliferation count in the AOX group compared with the CTRL group (Figure 4) using the pre-vaccination values as covariates (P < .05). Because of sample handling errors, these results were based on data from five animals. Interestingly, when stimulated with nonspecific mitogens such as PHA, lymphocyte proliferation was significantly higher for the CTRL group compared
with the AOX and GROC groups (Table 3). The data were not significantly different among these groups when stimulated with ConA or PWM, except for the WPI group, which was significantly higher than the GROC group when stimulated with ConA. These two sets of data showed that response to a specific antigen, one that was used as a vaccine, resulted in higher proliferation of specific memory lymphocytes, while the nonspecific proliferation of naïve and memory T cells in response to the nonspecific mitogen, under these conditions, was not significantly increased.

**Natural Killer Cell Activity**

There were no significant differences in the natural killer cell activity among the groups (data not shown).

**Complete Blood Counts and Serum Chemistries**

Serum vitamin E levels were significantly higher in the animals in the AOX and WPI groups compared with those in the CTRL and GROC groups (Table 4). Serum vitamin E values increased sixfold from the predietary intervention period in the AOX and WPI groups and twofold in the CTRL group. Animals in the GROC group had a twofold decrease in serum vitamin E levels after dietary intervention. Although there were some significant changes in serum chemistries, all values were within the normal ranges.

Using the predietary treatment
values as covariates, statistical analysis showed that the mean cell volume for the GROC group was significantly lower than that for all the other groups (Table 5). Between the pre- and postdietary treatments, erythrocytes and hemoglobin were significantly increased in the AOX and CTRL groups and hemoglobin was significantly increased in the WPI group. For the GROC group, there were no differences in blood counts between the pre- and postdietary intervention periods.

**DISCUSSION**

The main results from this study suggest that supplementation of vitamins E and C, β-carotene, and selenium supports the immune function responses examined in this study. This is reflected in the improved vaccination titers, increased circulating memory CD4+ cells, and improved lymphocyte response to the specific antigen in animals on the AOX diet. There were some significant differences in the serum chemistry values and complete blood counts, but all levels were within the normal range for puppies; body
weights and food intake were not significantly different among the groups. Vaccination titers were increased in all the groups after vaccination, although the titers for CPV decreased after week 4, the significant increases at weeks 2 and 3—indicate other changes in the immune cell populations, such as the memory T cells, which were also significantly elevated. Although a higher vaccination titer does not necessarily prove that immune function is better, what is known about memory T cell function suggests that increased levels of memory T cells will help to improve responses on reexposure to the antigens. This was indirectly shown in this study by the improved in vitro proliferation response of the lymphocytes to stimulation with the combined CDV and CPV (V7) antigens.

The actual mechanism for immune cell protection by antioxidants is still obscure, although it has been suggested that antioxidants such as vitamin E act on the signal transduction pathway leading to an inflammatory cascade. One of the possible mechanisms for the role of antioxidants may be protection of immune cells from free radical damage, as suggested by another study using similar vitamin E levels; the study showed that oxidative damage was reduced with supplementation of vitamin E.

Although a higher vaccination titer does not necessarily prove that immune function is better, what is known about memory T cell function suggests that increased levels of memory T cells will help to improve responses on reexposure to the antigens. This was indirectly shown in this study by the improved in vitro proliferation response of the lymphocytes to stimulation with the combined CDV and CPV (V7) antigens.

A deficiency in both vitamin E and selenium in the diet may lead to an inflammatory cascade. One of the possible mechanisms for the role of antioxidants may be protection of immune cells from free radical damage, as suggested by another study using similar vitamin E levels; the study showed that oxidative damage was reduced with supplementation of vitamin E.

Having a combination of antioxidants may spare other antioxidants, depending on the site and mechanism of actions of each. For example, the role of selenium in glutathione peroxidase may allow regeneration of glutathione, and may have a sparing effect on vitamin E or a powerful endogenous antioxidant in the body, such as the gastrointestinal tract. A deficiency in both vitamin E and selenium in the diet may lead to an inflammatory cascade. One of the possible mechanisms for the role of antioxidants may be protection of immune cells from free radical damage, as suggested by another study using similar vitamin E levels; the study showed that oxidative damage was reduced with supplementation of vitamin E.

Table 3. Lymphocyte Proliferation in Response to Mitogens (PHA, PWM, and ConA) before (Pre) and after (Post) Vaccination

<table>
<thead>
<tr>
<th>Diet</th>
<th>CTRL Pre</th>
<th>CTRL Post</th>
<th>AOX Pre</th>
<th>AOX Post</th>
<th>WPI Pre</th>
<th>WPI Post</th>
<th>GROC Pre</th>
<th>GROC Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA (1 µg/ml)</td>
<td>23,521 ± 8,540</td>
<td>42,554 ± 19,250</td>
<td>23,480 ± 12,826</td>
<td>25,713 ± 7,333</td>
<td>21,791 ± 13,685</td>
<td>35,177 ± 14,237</td>
<td>27,983 ± 10,871</td>
<td>25,744 ± 17,280</td>
</tr>
<tr>
<td>PWM (10 µg/ml)</td>
<td>23,862 ± 8,835</td>
<td>30,929 ± 18,992</td>
<td>20,420 ± 13,649</td>
<td>29,096 ± 11,603</td>
<td>22,945 ± 13,231</td>
<td>32,985 ± 12,955</td>
<td>25,393 ± 12,776</td>
<td>28,311 ± 17,465</td>
</tr>
<tr>
<td>ConA (2.5 µg/ml)</td>
<td>26,450 ± 11,845</td>
<td>43,192 ± 30,281</td>
<td>23,083 ± 16,928</td>
<td>46,522 ± 17,885</td>
<td>30,991 ± 15,886</td>
<td>20,630 ± 22,868</td>
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</tr>
</tbody>
</table>

Values are counts/min (cpm) ± SD of the stimulated means after deducting the unstimulated means.

Lymphocytes from the group on the CTRL food had significantly higher proliferation when stimulated with PHA than the groups on the AOX and the GROC foods. Lymphocyte proliferation was also significantly higher for the group on the WPI diet compared with those on the GROC diet when stimulated with ConA.

AOX = antioxidant diet; ConA = concanavalin A; CTRL = control diet; GROC = grocery brand diet; PHA = phytohemagglutinin; PWM = pokeweed mitogen; WPI = whey protein diet.
### TABLE 4. Serum Vitamin E Values in Puppies before (Day 1 [Pre]) and after (Day 42 [Post]) Dietary Intervention (Mean ± SD)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CTRL</th>
<th>AOX</th>
<th>WPI</th>
<th>GROC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre Post</td>
<td>Pre Post</td>
<td>Pre Post</td>
<td>Pre Post</td>
</tr>
<tr>
<td>Serum vitamin E (µg/ml)</td>
<td>12.54 ± 2.84 20.71&lt;sup&gt;a&lt;/sup&gt; ± 3.66 11.52 ± 5.04 32.45&lt;sup&gt;b&lt;/sup&gt; ± 4.45 12.68 ± 3.34 40.15&lt;sup&gt;c&lt;/sup&gt; ± 9.31 13.35 ± 3.85 6.73&lt;sup&gt;d&lt;/sup&gt; ± 1.6</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

<sup>a,b,c,d</sup> All serum vitamin E levels were significantly different from each other after dietary intervention. There were no significant differences in the serum vitamin E levels before dietary intervention. The data showed that serum vitamin E levels increased threefold in the AOX and WPI groups and twofold in the CTRL group but decreased twofold in the GROC group (<i>P</i> < .05) following dietary intervention.

AOX = antioxidant diet; CTRL = control diet; GROC = grocery brand diet; WPI = whey protein diet.

### TABLE 5. Complete Blood Count Results for Puppies before (Day 1 [Pre]) and after (Day 42 [Post]) Dietary Intervention

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CTRL</th>
<th>AOX</th>
<th>WPI</th>
<th>GROC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre Post</td>
<td>Pre Post</td>
<td>Pre Post</td>
<td>Pre Post</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>11.8 ± 1.7 13.7 ± 4.1 13.1 ± 3.7 13.1 ± 2.6 13.9 ± 6.0 14.6 ± 3.3 13.6 ± 3.1 13.7 ± 2.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>4.7 ± 0.3&lt;sup&gt;x&lt;/sup&gt; 5.0 ± 0.3&lt;sup&gt;y&lt;/sup&gt; 4.9 ± 0.3&lt;sup&gt;x&lt;/sup&gt; 5.3 ± 0.4&lt;sup&gt;y&lt;/sup&gt; 4.7 ± 0.3 5.0 ± 0.04 5.1 ± 0.3 5.2 ± 0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>10.2 ± 0.5&lt;sup&gt;x&lt;/sup&gt; 11.4 ± 0.6&lt;sup&gt;y&lt;/sup&gt; 11.1 ± 0.9&lt;sup&gt;x&lt;/sup&gt; 12.0 ± 0.8&lt;sup&gt;y&lt;/sup&gt; 10.3 ± 0.7&lt;sup&gt;x&lt;/sup&gt; 11.3 ± 1.0&lt;sup&gt;y&lt;/sup&gt; 11.4 ± 0.7 11.7 ± 0.5</td>
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<tr>
<td>Hematocrit</td>
<td>32.7 ± 1.5 34.9 ± 1.5 35.3 ± 3.1 36.8 ± 2.5 33.0 ± 2.2 34.7 ± 3.0 36.3 ± 2.0 36.1 ± 1.4</td>
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<tr>
<td>Mean cell volume</td>
<td>70.0 ± 2.6 70.2 ± 1.6&lt;sup&gt;a&lt;/sup&gt; 65.2 ± 20.7 70.2 ± 1.8&lt;sup&gt;a&lt;/sup&gt; 70.3 ± 2.2 69.9 ± 1.9&lt;sup&gt;a&lt;/sup&gt; 71.3 ± 2.6 67.3 ± 2.6&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

<sup>a,b</sup> Different superscripts denote significantly different values among the treatment groups in the same treatment period (<i>P</i> < .05).<br>
<sup>x,y</sup> Different superscripts denote significantly different values between the pre- and postdietary intervention period within each treatment group (<i>P</i> < .05).<br>

AOX = antioxidant diet; CTRL = control diet; GROC = grocery brand diet; WPI = whey protein diet.
dogs also resulted in a severe suppression of lymphocyte proliferative response to mitogens compared with a moderate reduction when the dogs were fed food deficient in either one alone.\textsuperscript{31} Our study contrasts with the other because the lymphocyte proliferation was not suppressed in the supplemented dogs but rather was increased in the nonsupplemented dogs. However, the levels in our study were not considered deficient as in the study by Lessard and colleagues.\textsuperscript{31} Another study looking at β-carotene supplementation in dogs also found that lymphocyte proliferation was not affected, although delayed-type hypersensitivity skin response to vaccine and PHA was increased.\textsuperscript{32}

There were some trends in the data that did not reach significance; this may be a function of the amount of antioxidants in the CTRL and GROC diets, which were formulated to meet the nutrient requirements of dogs (AAFCO 2004), and the small number of animals in this study (N = 10/group). The true requirements for the antioxidant function of the vitamins under conditions of stress are not known. The levels in all the food may help alleviate some of the oxidative stress in the body. Serum vitamin E levels, which were significantly increased in the AOX and WPI groups, increased slightly or did not change in the CTRL group and actually decreased in the GROC group during the study, suggesting that vitamin E levels in the GROC food (70 IU/kg) may not be sufficient to maintain serum levels when faced with the vaccination challenges to the immune system. An interesting observation about antioxidants is their mobilization to the site of free radical attack. A review paper described a study in rats showing that serum and lung levels of vitamin E increased as a result of increased mobilization when they were exposed to nitric oxide (NO\textsubscript{2}) and other environmental pollutants.\textsuperscript{33} The increase was significant only in supplemented rats, suggesting that oxidative stress resulted in an increased demand on the body stores and that the body stores needed to be supplemented sufficiently to cope with the stress.

A feature of the improved immune response in puppies noted in the present study was that there did not appear to be a widespread nonspecific immune response to the vaccination, as suggested by a smaller increase to nonspecific mitogen stimulation, such as with ConA, PHA, or PWM in the AOX group. The re-

\textbf{There did not appear to be a widespread nonspecific immune response to the vaccination.}
This could mean that puppies are less likely to develop the aberrant inflammatory response that is the root of many inflammatory diseases (e.g., inflammatory bowel disease) and allergies. However, the low level of response to mitogen stimulation in the GROC group suggested that the immune cells may be hyporesponsive, possibly as a result of vaccination stress, and thus unable to mount a strong response to nonspecific mitogen or specific antigen stimulation.

The test food containing both whey protein isolate and antioxidants (WPI diet) did not show much of an additive effect over the AOX formula and, in fact, may have adversely affected the benefits seen with the inclusion of antioxidants. Although the levels of antioxidants in the WPI food were similar to those in the AOX food (as shown by the dietary analysis and the serum vitamin E levels), the vaccination response and the lymphocyte proliferation was more similar to that seen in the CTRL group. Without knowing the actual components of the whey protein that survived processing and was active in the food, it is difficult to speculate on the effect of the whey protein alone or the effect of the interaction between the whey protein and the antioxidants on the immune response in the body in this study.

I CONCLUSION

During the 6-week study, the combination of antioxidants used in this study—vitamin E (500 IU/kg DM), vitamin C (70 mg/kg DM), β-carotene (0.4 mg/kg DM), and selenium (0.8 mg/kg DM)—was found to significantly increase vaccination titers in response to CDV compared with the other foods. CPV titers were also increased in the AOX group during the study period but were similar to those in the other groups by week 4 after vaccination. The AOX diet also increased the number of memory T cells, which are thought to confer immunologic memory. This increase may be responsible for the higher in vitro lymphocyte proliferative response to the specific viral antigen used in the vaccine but not to a nonspecific mitogen in the AOX group. The minimum AAFCO recommendation for vitamin E in puppies (50 IU/kg) may not be sufficient to allow optimal response for the cells during periods of immune stress.

REFERENCES


