INTRODUCTION

Dietary carotenoids have been shown to help protect the immune system from oxidative damage resulting in an enhanced cell-mediated immune response. It has been demonstrated in multiple species that carotenoids are taken up in the blood and leukocyte subcellular components, including β-carotene in cattle (Chew et al., 1993), pig (Chew et al., 1991a,b), and dogs (Chew et al., 2000a) as well as lutein (Chew et al., 1998; Kim et al., 2000) and astaxanthin (Park et al., 2010) in dogs and cats. Bioavailability and bioconversion of carotenoids depends on many factors, including structure and molecular linkages and concentration administered (Castenmiller and West, 1998).

Bixin, the primary carotenoid found in the seed pericarp of the Annatto plant, *Bixa orellana*, is used commercially as a food colorant and has been a

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Uptake and immunomodulatory role of bixin in dogs

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ABSTRACT: Carotenoids are readily absorbed from the diet and distributed in blood leukocyte subcellular organelles. Bixin, a potent bioactive found in the seed of the Annatto plant, *Bixa orellana*, possesses antioxidant and anti-inflammatory properties. The purpose of this study was to determine the uptake of bixin by plasma, lipoproteins, and leukocytes in domestic dogs and to examine immunoprotective properties. To determine uptake kinetics, female Beagle dogs (2 yr; 9.1 ± 0.1 kg BW) were first fed a single dose by oral gavage of 0, 5, 10, 20, or 40 mg bixin, with blood collected at 0 to 16 h after administration (*n* = 6/treatment), and then fed daily with 0, 5, 10, 20, or 40 mg bixin/d, with blood collected at 0, 1, 2, 4, 6, 10, and 14 d. In a consecutive experiment, cell-mediated and humoral responses as well as oxidative biomarkers were measured following 16 wk of dietary supplementation with 0, 5, 10, or 20 mg bixin/d. Maximal absorption in plasma occurred by 0.5 h with an elimination half-life of 2.6 to 3.3 h after a single dose of bixin. Steady-state plasma concentrations were 0.053 μM after 14 d of 40 mg bixin/d. The majority of subcellular bixin was found in the leukocyte mitochondria and was associated with the high-density lipoprotein and low-density lipoprotein fractions of lipoproteins. Specific (vaccine) response increased (*P* < 0.05) but nonspecific mitogen response was unchanged after 12 wk of dietary bixin, as assessed by a delayed-type hypersensitivity assay. Both B cell plasma leukocyte subpopulations at 6 and 16 wk and IgG plasma concentration at 12 wk in the 10-mg treatment group increased (*P* < 0.05), although IgM production and other cell populations were unaffected. In addition, 8-oxo-2′-deoxyguanosine (8-OHDG), a DNA damage biomarker, was substantially reduced (*P* < 0.05) in all treatment groups by wk 16, and C-reactive protein (CRP) was suppressed at wk 12 (*P* < 0.05). Dietary supplementation with bixin showed no changes in lymphoproliferation in response to in vitro mitogenic challenge and had no effect in enhancing natural killer cell activity. In conclusion, bixin was readily absorbed in a dose-dependent manner in blood following oral administration and was then taken up by leukocytes, where it was primarily distributed to mitochondria but in other subcellular organelles as well. Bixin also appeared to stimulate immune response, as seen with cell-mediated responses, and exerted anti-inflammatory (reduced CRP) as well as antioxidative (reduced 8-OHdG) effects in dogs.

Key words: bixin, canine, immunomodulation, oxidative biomarkers, uptake kinetics


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common ingredient in the Latin American diet for centuries (Evans, 2000). It is unique among the naturally occurring carotenoids due to its 2 carboxylic groups, one of which is a methyl ester (Levy et al., 1997), which makes bixin much more polar than most carotenoids. Bixin exerts strong singlet molecular oxygen quenching activity (Di Mascio et al., 1990) and is a potent inhibitor of lipid peroxidation (Zhang et al., 1991; Canfield and Valenzuela, 1993), thereby protecting cell membranes from oxidation. The structure of bixin allows uptake into leukocytes, which may increase bioavailability and, therefore, enhanced immune effects. Although bixin is the predominant carotenoid extracted from the Annatto seed, making up 70 to 80% of the pigment present, norbixin is also present in small amounts and is the most abundant water-soluble derivative following hydrolysis of bixin (Barcelos et al., 2012).

It is hypothesized that oral bixin supplementation will be readily taken up in plasma in dogs and intracellularly in leukocytes and will enhance immunomodulation. Our present objectives are 1) to quantitate uptake of bixin and norbixin in plasma, partitioning in blood-associated lipids, and subcellular distribution when given orally to Beagle dogs and 2) to assessing stress and inflammatory biomarkers to assess immunomodulatory and antioxidant effects.

MATERIALS AND METHODS

The research protocol was approved by the Washington State University Institutional Animal Care and Use Committee (number 3229).

Animals and Experimental Design

Female Beagle dogs (2 yr; 9.1 ± 0.1 kg BW; Marshall Farms USA, Inc., North Rose, NY) were fed a nutritionally balanced diet (200 g∙dog∙d; P&G Pet Care, Lewisburg, OH) with a composition of 66.2 g/kg moisture, 262 g/kg protein, 74.5 g/kg ash, 160 fat g/kg, 14.8 g/kg Ca, 10.3 P g/kg, and 437.3 g/kg nitrogen-free extract. Dogs (total n = 56) were housed in 2 by 2 m pens (2 dogs/pen) in a temperature- (20 to 22°C) and light- (14 h light) controlled facility. Uptake kinetics and subcellular distribution were established in an initial experiment consisting of 2 parts. Acute uptake was examined by giving dogs (n = 6/treatment group) a single dose of 0, 5, 10, 20, or 40 mg bixin by oral gavage, and blood was taken at 0, 0.5, 1, 2, 4, 8, and 16 h after feeding. Steady-state uptake, subcellular distribution, and lipoprotein-associated distribution were determined with oral doses of 0, 5, 10, 20, or 40 mg bixin (n = 6/treatment) administered daily at 0800 h for 14 consecutive days, with blood collection 1 h after each feeding on d 0, 2, 4, 6, 10, and 14.

To examine immune responses to dietary bixin, dogs were randomly assigned (n = 14/treatment) to be fed 0, 5, 10, or 20 mg bixin daily for 16 wk. Because the uptake kinetic studies were short term, the 40-mg dose was included to provide a wider range of uptake kinetics. However, with the 16-wk immune study, because supplementation was for a much longer period, in addition to the fact that the 20-mg dose provided very significant uptake in the blood, it was determined that the 40-mg dose, which is a nonphysiologic dose, would not significantly contribute to overall interpretation of immune response. Blood was collected by jugular venipuncture into vacutainers containing heparin or EDTA on wk 0, 6, 12, and 16 for immune function assessment. All dogs were vaccinated with an attenuated polyvalent vaccine containing canine distemper virus, adenovirus type-2, parainfluenza virus, and parvovirus (Vanguard 5; SmithKline Beecham Animal Health, West Chester, PA) on wk 12 and 14 and blood was collected on wk 16 to assess postvaccination immune responses.

Bixin Preparation

A stock suspension of bixin in soybean oil was prepared from annatto seed extract (17 g/L in soybean oil containing 1.6% bixin and <0.3% norbixin; INEXA, Quito, Ecuador) and warmed at 60°C for 15 min, and then bixin treatment solutions were diluted in soybean oil for a final dosage volume of 1 mL (Levy et al., 1997).

Plasma and Cellular HPLC Analysis

Blood was collected into heparinized vacutainers and centrifuged at 400 × g for 30 min at 4°C, and plasma was collected, aliquoted, and stored at −80°C. Theuffy coat leukocytes were collected on 6 and 14 d, lymphocytes were enriched using density gradient (Histopaque-1077; Sigma-Aldrich, St. Louis, MO) centrifugation room temperature (RT; Chew et al., 1993), and the number was determined using a Coulter counter (Coulter Electronics, Hialeah, FL). Cells were then resuspended in PBS containing 30 g/L sodium ascorbate (Sigma-Aldrich) to prevent oxidation and disrupted by sonication (30 s). On d 14, the whole lymphocyte bixin concentration was determined by HPLC analysis after extraction of bixin from an aliquot of the lymphocyte homogenate. Subcellular fractions were also prepared from cell homogenates as previously described (Chew et al., 1993). Briefly, homogenates were differentially centrifuged to obtain nuclear (600 × g for 10 min at 4°C), mitochondrial (17,300 × g for 20 min at 4°C),
Bixin uptake and immunomodulation in dogs

Plasma, lymphocyte homogenate, and lymphocyte subcellular fractions were analyzed for bixin content by HPLC (Alliance 2690; Waters Corp., Milford, MA) as previously described (Levy et al., 1997) with modification. Sudan I has solubility characteristics similar to bixin and norbixin, thereby removing the need for quantitative solvent transfer during the extractions, and was therefore used as an internal standard at 3.2 μM Sudan I (Sigma-Aldrich) in methanol. Sudan I (0.1 mL) and plasma (0.3 mL) were vortexed for 1 min and then incubated in the dark for 30 min. Chloroform (0.6 mL) was added and samples were vortexed for 5 min and then centrifuged 10 min at 1,000 × g at 4°C. The bottom layer was collected and dried under nitrogen gas in a water bath (40°C). The resulting residue was resuspended in a mobile phase consisting of acetonitrile:2% acetic acid in water (70:30, vol/vol), samples were eluted through a 5-μm silica C18 column (250 by 4.6 mm, Spherisorb; Waters Corp), and absorbance was monitored at 460 nm (Photodiode Array Detector 996; Waters Corp.).

Lipoprotein Uptake and Cholesterol Analysis

After centrifugation (400 × g for 30 min at RT) of blood collected into vacutainers containing no anticoagulants, lipoproteins were isolated from serum using density gradient ultracentrifugation (Terpstra et al., 1981; Chew et al., 1993). Resulting lipoprotein fractions were removed and analyzed for bixin by HPLC and the cholesterol content was determined (Chew et al., 1993).

Hematology and Leukocyte Subsets

Ethylenediaminetetraacetic acid–treated whole blood was analyzed for complete blood count (white blood cell, red blood cell, and platelet counts; lymphocyte, monocyte, and granulocyte differential counts; hematocrit, hemoglobin, and mean corpuscular volume; hemoglobin and hemoglobin concentration; and platelet volume) using a hematology analyzer (Vet ABC-Hematology Analyzer; Heska Corp., Fort Collins, CO).

Leukocytes were prepared from whole blood after lysing red blood cells using hypotonic shock followed by centrifugation at 300 × g for 5 min at 20°C. After resuspension in PBS, pH 7.3, containing 10% fetal bovine serum and 0.02% sodium azide, cells were stained as previously described (Kim et al., 2000). Monoclonal antibodies (Leukocyte Antigen Biology Laboratory, Davis, CA) against specific subpopulations included CD3+ (CA17.2A12, total T cells), CD4+ (CA13.1E4, helper T cells [Th]), CD8+ (CA9. JD3, cytotoxic T cells [Te]), CD18+ (CA16.3C10, an adhesion molecule), and CD21+ (CA2.1D6, mature B cells). Changes in subpopulations of cells on wk 0, 6, 12, and 16 were quantitated by flow cytometry (FACSCaliber; BD Biosciences, San Jose, CA).

Plasma Immunoglobulin Analysis

B cell function in the humoral immune response was assessed by measuring concentrations of plasma IgG and IgM using commercially available ELISA kits (Dog IgG ELISA Quantitation Kit and Dog IgM ELISA Quantitation Kit; Bethyl Laboratories, Montgomery, TX). The lower limits of detection were 7.8 and 15.6 ng/mL for IgG and IgM, respectively.

Leukocyte Immune Responsiveness

Delayed-type hypersensitivity was assessed by measuring skin induration response in all dogs on wk 12 and 16. Dogs were intradermally injected with physiologic saline (negative control), vaccine (to elicit a specific response to recognized antigens), and phytohemagglutinin (PHA; 0.5 g/L saline), as previously described (Kim et al., 2000). Skin thickness was measured at 0, 24, 48, and 72 h after injection, and results were expressed as percentage of increase in skin thickness compared with h 0.

The in vitro proliferation responses of peripheral blood mononuclear cells to the T cell mitogens (Sigma-Aldrich), PHA (2 and 10 mg/L final concentration) and concanavalin A (ConA; 1 and 5 mg/L) as well as the T and B cell mitogen derived from pokeweed mitogen (0.25 and 1.25 mg/L) were assessed (Kim et al., 2000), with modifications. Whole blood was cultured to mimic in vivo conditions. Heparinized blood collected on wk 0, 6, 12, and 16 was diluted 1:8 in RPMI-1640 (developed at Roswell Park Memorial Institute, Buffalo, NY) supplemented with 10% newborn calf serum and penicillin–streptomycin (Sigma-Aldrich). Resulting cpm in harvested cells were expressed as a stimulation index (SI), calculated as the percent proliferation compared with unstimulated controls.

Natural killer cell cytotoxicity against tumor cells (ATCC, Manassas, VA) was assessed using a modified bioassay (Heo et al., 1990; Chew et al., 2011). Canine thyroid adenocarcinoma cells, used as target cells, were plated in 96-well flat-bottom culture plates at 1 × 10^4 cells/well in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. Peripheral blood mononuclear cells, which acted as effector cells, were prepared by lysing whole blood with ammonium-chloride-potassium buffer (150 mM...
NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA, pH 7.2). Peripheral blood mononuclear cells were resuspended to 1 x 10⁶ and 2 x 10⁶ cells/mL and 100 µL/well was added to the target cells to provide effector:target ratios of 10:1 and 20:1. Following overnight incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0.1 mg/well) was added and incubated for 4 h, the supernatant was removed, and the formazan was resuspended in isopropanol. Optical density was measured at 550 nm and the percent of specific cytotoxicity was calculated.

**Inflammatory and Oxidative Damage Biomarkers**

C-reactive protein (CRP), an acute phase protein, was measured in plasma using a sandwich ELISA (PHASE Canine CRP Assay Kit; Tri Delta Diagnostics, Morris Plains, NJ), with a lower limit of detection of 7.5 ng/mL. This parameter was assessed as a biomarker to evaluate anti-inflammatory properties of bixin.

Deoxyribonucleic acid oxidative damage was assessed in plasma by measuring 8-hydroxy-2'-deoxyguanosine (8-OHdG) using a competitive ELISA (BIOXYTECH 8-OHdG-EIA; Percipio Biosciences, Burlingame, CA), with a lower limit of detection of 0.5 ng/mL. This parameter was assessed as a biomarker to evaluate antioxidant properties of bixin.

**Statistical Analysis**

Data were analyzed by repeated sampling ANOVA using the General Linear Models of SAS (SAS Inst. Inc., Cary, NC). The statistical model was Yijk = µ + Treatmenti + Animalj(Treatmenti) (error A used to test the effects of treatment) + Sampling periodk + Treatmenti x Periodj + εijk (error B). Differences among treatment means within a sampling period were compared by a protected LSD test.

**RESULTS**

**Uptake in Plasma and Subcellular Organelles**

Bixin was undetectable in the plasma of unsupplemented dogs during the entire study period as well as in all dogs before bixin supplementation. Uptake of bixin into plasma was rapid, with maximal absorption occurring with 0.5 h. Maximal plasma bixin absorption was low (0.006 ± 0.001 µM) at 0.5 h in dogs fed 5 mg in a single dose of bixin. Compared with the 5-mg group, maximal absorptions 0.5 h after dosing in dogs fed a single dose of 10 (0.011 ± 0.003 µM), 20 (0.053 ± 0.011 µM), or 40 mg bixin (0.080 ± 0.016 µM) were 2-, 10-, and 13-fold greater (P < 0.05), respectively. All treatment groups showed decreased plasma final concentrations of less than 0.006 µM by 8 h. The half-life of bixin in the plasma was approximately 2.6 h in the 10- and 20-mg groups and 3.3 h in the 40-mg group. Plasma norbixin, on the other hand, showed very little uptake following the single dose of bixin, with an increase to 0.002 M by 8 h in the 40-mg treatment group.

Plasma bixin concentrations also increased (P < 0.05) in a dose-dependent manner when administered daily (Fig. 1). Concentrations increased (P < 0.05) after the first dose and maintained fairly stable steady-state concentrations through d 14 of 0.007 ± 0.002, 0.024 ± 0.010, 0.032 ± 0.003, and 0.053 ± 0.007 µM for the 5-, 10-, 20-, and 40-mg groups, respectively. Dogs fed 10, 20, and 40 mg bixin averaged 4 to 9 times greater (P < 0.05) plasma bixin when compared with those fed 5 mg bixin. Dietary bixin did not influence plasma α-tocopherol and retinol concentrations, and averaged 2.297 ± 0.218 and 0.034 ± 0.001 µM, respectively.

Bixin was not detectable in pooled whole lymphocyte homogenates in control dogs but increased in a dose-dependent manner in all treatment groups on both d 6 and 14 (Fig. 1), with a 5-fold increase in bixin accumulation on d 14 compared with d 6. Fractionation of the peripheral blood lymphocytes showed an initial subcellular bixin uptake on d 6 primarily in the mitochondrial fraction in all treatment groups (75–85% of total whole leukocyte bixin in the 10-, 20-, and 40-mg treatment groups), although total uptake in the 5-mg treatment group was low (13.6 pmol/10⁶ leukocytes). This was also the case with distribution of bixin in the microsomal fraction, albeit at a much smaller percentage (9–11% in the 10-, 20-, and 40-mg treatment groups). On d 14, bixin distribution was again highest in the mitochondrial fraction in all treatment groups (61–82% of total cellular bixin) but was also substantial in the microsomal fractions of all treatments as well (10–32% of total cellular bixin). Nuclear accumulation of bixin was apparent in the highest doses of bixin, with distribution comparable to that of the microsomal fraction.

Bixin was not detectable in any lipoprotein fraction in unsupplemented dogs but was taken up by high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very-low-density lipoprotein (VLDL) in a dose-related manner in all treatment groups on both d 6 and 14 (Fig. 2) and was highest (P < 0.05) in the 40-mg treatment group. Bixin associated with the blood HDL and LDL fractions was equivalent on both d 6 and 14 in the 5-, 10-, and 20-mg treatment groups. Although bixin associated with HDL was equivalent to that with the LDL fraction on d 6, the HDL fraction had greater bixin (P < 0.05) on d 14. Bixin associated with HDL and LDL fractions was 3 to 4 and 5 to 7 times greater than the VLDL fraction on d 6 and 14, respectively.
Bixin uptake and immunomodulation in dogs

Hematology results following 16 wk of bixin treatment are seen in Table 1. Bixin increased (P < 0.05) percent granulocytes (including basophils, eosinophils, and neutrophils) in the 10-mg treatment group and tended to be greater in the 20- and 5-mg treatment groups as well when compared with the control. No other differences were observed in the hematology parameters examined.

The distribution of different lymphocyte subpopulations is shown in Table 1. As seen with the percent lymphocytes in the hematology results, CD3+ Tc cell relative percentage decreased with the 10-mg bixin treatment compared with the control. In addition, there was an increase (P < 0.05) in relative percentage of CD21+ B cell subpopulations of lymphocytes at both wk 6 and 16 and a tendency for an increase at wk 12 in the 10-mg treatment group. There were no treatment differences in either CD4+ Th (overall mean [SEM] of 51.0 [1.0], 48.5 [1.2], 52.4 [2.2], and 47.3 [1.0] at wk 0, 6, 12, and 16, respectively) or CD8+ Tc subpopulations (overall mean [SEM] of 2.6 [0.3], 3.0 [0.3], 8.3 [1.3], and 6.6 [0.5] at wk 0, 6, 12, and 16, respectively). When the overall population of leukocytes was examined, there appeared to be a decrease (P < 0.05) in CD18+ cells at wk 6 within the 20-mg treatment group when compared with the 0- and 5-mg treatment groups. There was no difference in the CD18+ granulocyte subpopulation; however, there was a decrease (P < 0.05) in the CD18+ lymphocyte subpopulation in the 10-mg treatment group when compared with the 0-mg treatment group.

Plasma IgG and IgM

Concentrations of plasma IgM (overall mean [SEM] of 1.36 μg/mL [0.07]) and IgA (overall mean [SEM] of 0.56 mg/mL [0.02]) did not show changes in concentrations throughout 16 wk of treatment with dietary bixin. Plasma IgG, on the other hand, increased (P < 0.05) at wk 12 in the 10-mg treatment group compared with the 0-mg treatment group (Table 1). This increase paralleled the increase in CD21+ B cell subpopulations seen at wk 12 in the 10-mg treatment group.

Figure 1. Concentrations of plasma bixin and bixin uptake in subcellular fractions (nuclear, microsomal, mitochondrial and cytosolic) in dogs administered daily doses of 0, 5, 10, 20 or 40 mg bixin for 14 d. Values are means ± SEM (n = 6) as analyzed by repeated measures ANOVA. Means with different superscripts within a sampling period are significantly different (P < 0.05). nd = non-detectable.

Figure 2. Concentrations of bixin in whole lymphocytes and lipoprotein fraction in dogs given a single oral dose of 0, 5, 10, 20 or 40 mg bixin for 14 d. Values are means ± SEM (n = 8) as analyzed by repeated measures ANOVA. Means with different superscripts within a sampling period are significantly different (P < 0.05). nd = non-detectable.
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Table 1. Hematology and phenotyping of blood leukocytes at wk 1, 6, 12, and 16 in dogs fed 0, 5, 10, or 20 mg bixin daily. Plasma IgG reflected B cell function. Values are means (SEM; n = 14)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Week</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>20</th>
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<tr>
<td>WBC, 10^9/mm^3</td>
<td>16</td>
<td>8.0 (0.2)b</td>
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<td>7.6 (0.4)b</td>
<td>8.5 (0.8)ab</td>
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<td>Lymphocyte, %</td>
<td>16</td>
<td>17.8 (1.1)a</td>
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<td>15.6 (0.6)b</td>
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<td>5.3 (0.2)ab</td>
<td>4.7 (0.3)c</td>
<td>4.9 (0.2)bc</td>
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<td>Granulocyte, %</td>
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<td>78.4 (1.0)ab</td>
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<td>Relative percent</td>
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<td>CD3+ Total</td>
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<td>74.2 (2.3)</td>
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<td>77.8 (2.9)ab</td>
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<td>80.4 (1.9)ab</td>
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<td>16</td>
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<td>17.6 (5.4)ab</td>
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a–c Differences within variable between treatments (P < 0.05).

**Leukocyte Immune Responsiveness**

Bixin stimulated (P < 0.05) the delayed-type hypersensitivity induration response to vaccine at 12 wk in the 20-mg treatment group (Table 2) at 48 and 72 h following intradermal challenge with vaccine. Additionally, bixin had no effect on response at 24 and 48 h (mean [SEM] of 43% [2] and 27% [2] increase, respectively) following challenge with PHA. Skin thickness increased (P < 0.05) at 72 h after challenge with PHA only in the 5-mg group compared with the control and 10- and 20-mg treatment groups. Conversely, at 16 wk, the 5-mg treatment group actually showed a depressed response (P < 0.05) compared with the control treatment group (Table 2) at 48 and 72 h following challenge with PHA. There was little difference in plasma CRP concentrations at wk 12 but by 16 wk were dramatically suppressed (P < 0.05) by all treatment levels (Fig. 3) when compared with the 0-mg control treatment.

**Inflammatory and Oxidative Biomarkers**

After 16 wk of dietary bixin supplementation, plasma 8-OHdG levels, indicating DNA oxidative damage, were unaffected for 12 wk but by 16 wk were dramatically suppressed (P < 0.05) by all treatment levels (Fig. 3) when compared with the 0-mg control treatment. Plasma CRP concentrations at wk 12 were lower (P < 0.05) in all treatment groups (Fig. 3) when compared with the control. There was little difference in plasma CRP concentrations (overall mean [SEM]) at wk 0 (7.3 mg/mL [0.9]), 6 (2.4 mg/mL [0.7]), or 16 (5.9 mg/mL [0.7]).

**DISCUSSION**

It is readily apparent that uptake and intracellular distribution following oral supplementation with carotenoids varies greatly, dependent on the nature of the compounds (Sy et al., 2012) and among species. Little is known concerning the uptake and metabolism in dogs administered oral bixin, and the effects of absorption and bioconversion will influence bioavailability (Castenmiller and West, 1998). Levy et al. (1997) demonstrated measurable levels...
of bixin and norbixin in the blood following ingestion of a single dose of Annatto food color in humans. It has also been demonstrated that norbixin can be distributed in cells, because it protects DNA from oxidative damage, and these hydroxyl radicals cannot diffuse from their production site (Kovary et al., 2001). Along with bixin, norbixin exerts protective effects against mercury-induced DNA damage (Kovary et al., 2001; Barcelos et al., 2012) and exerts antimutagenic properties (Júnior et al., 2005).

In this study, domestic dogs were able to absorb orally administered bixin (0.14 μM plasma peak concentration), but norbixin absorption was extremely low (<0.005 μM). In addition, bixin was rapidly taken up in dog plasma, with peak absorption occurring within 1 h. This uptake occurs earlier than that of humans given oral bixin (2 h; Levy et al., 1997) as well as with dogs fed astaxanthin (3–6 h; Park et al., 2010) or β-carotene (6–12 h; Chew et al., 2000a). Although plasma elimination half-life in dogs is similar between bixin (2.6–3.3 h) and β-carotene (3–4 h; Chew et al., 2000a), it is a much shorter interval than that seen with astaxanthin uptake (Park et al., 2010). On the other hand, a dosage of 1 mg carotenoid/kg BW was similar (0.05 μM) in dog plasma for both astaxanthin and β-carotene when compared with bixin.

Following absorption in the intestinal mucosa, carotenoids are incorporated into chylomicrons for transport to the liver, thereby allowing for either storage or subsequent secretion from the liver in VLDL (Olson, 1994). In human blood, astaxanthin was found in all lipoprotein fractions with the majority (64%) distributed in the VLDL fraction, but the remaining astaxanthin (36%) was evenly distributed between the HDL and LDL (Osterlie et al., 2000). Similar results were found with β-carotene uptake in humans, with 70% distributed in the VLDL and the remaining 30% evenly distributed between the HDL and LDL (Romanchik et al., 1994). High-density lipoproteins are the major lipoproteins in the blood of dogs, so it is not surprising that the only lipoprotein with measurable astaxanthin in dogs was the HDL fraction (Chew et al., 2000a). However, bixin associated comparably with HDL and LDL (39 and 44%, respectively) and with measurable bixin in the VLDL fraction (17%) on both d 6 and 14. This is consistent with the distribution of polar xanthophylls, generally evenly distributed between HDL and LDL, which differ from nonpolar carotenoids transported primarily in LDL (Goulinet and Chapman, 1997).
centrations of bixin in the mitochondria may be important in preventing damage to membranes because mitochondria are a significant source of reactive oxygen species (Shigenaga et al., 1994) and carotenoids reduce free radical formation in lipid membranes (Machlin and Bendich, 1987). In fact, bixin may be more effective in maintaining normal mitochondrial function and membrane fluidity than astaxanthin and β-carotene; when compared with mitochondrial concentrations of bixin, astaxanthin (43–50%; Park et al., 2010) and β-carotene (17%; Chew et al., 2000a) were lower.

The contribution of carotenoids to immune health through immunoregulatory action and anticancer effects is well known, and bixin, a naturally available carotenoid, has also been shown to have a beneficial impact on these functions as well. Specifically, the carotenoids canthaxanthin and β-carotene have been shown to reverse neoplastic transformation in a mouse embryo cell line by up-regulating connexin gene expression, thereby increasing intercellular communication via gap junctions (Bertram, 2004), which is important in regulating cell growth and differentiation. In contrast, although canthaxanthin also increased gap junctional communication in primary human fibroblasts, astaxanthin caused a decrease (Daubrawa et al., 2005). Bixin also did not upregulate connexin gene expression (Zhang et al., 1991), but it has been shown that it is able to induce cytotoxicity in multiple human cancer cell lines (Tibodeau et al., 2010) and decrease aberrant crypt foci, precursors of colon cancer, if administered before the induction phase (Agner et al., 2005). In addition, bixin was able to decrease some of the chromosomal damage induced by the chemotherapeutic cisplatin and actually increase proliferation of human lymphocytes put in culture (Antunes et al., 2005) as well as decreasing growth of rat adrenal neuroendocrine tumors while decreasing cisplatin-induced reactive oxygen species (dos Santos et al., 2012). A similar decrease in tumor development was also seen in mice supplemented with the carotenoid lutein (Chew et al., 2003).

Immunomodulatory effects of bixin, as well as other carotenoids, are also well documented. Stimulation of cell-mediated and humoral response by dogs has been demonstrated with β-carotene (Chew et al., 2000b), lutein (Kim et al., 2000), and astaxanthin (Chew et al., 2011). In this study, bixin also increased a nonspecific as well as specific cell-mediated response on antigenic challenge. However, unlike astaxanthin and lutein, there was no change in lymphocyte proliferation in response to mitogens, indicating these carotenoids have differential effects on immunoregulation. There was a decrease in total lymphocytes when dogs were fed bixin, in contrast to the increases seen in total T cell, Th, and Tc subpopulations when dogs were fed lutein or β-carotene (Chew et al., 2000b; Kim et al., 2000). There was a definite increase in B cell populations, which is consistent with that seen with astaxanthin supplementation, and as with all of these carotenoids, there was an increase in plasma IgG.

Peripheral blood phagocytic cell CD18+ adhesion molecule expression increases during systemic inflammation (Kallio et al., 2008). However, in this study, there was a decrease in CD18+ cell subpopulations after dietary supplementation with bixin, demonstrating one type of anti-inflammatory activity. Oral supplementation with bixin was shown to counter inflammatory effects in rats when paw edema was induced by subcutaneous injection of bradykinin (Yoke Keong et al., 2011). Although not significant, there was a slight anti-inflammatory effect of bixin at wk 12, as seen in the decreased plasma CRP concentrations. Humoral effects were not examined in this study but might provide additional indications of suppression of inflammation in response to bixin.

Bixin also showed antioxidant effects, as seen with the decreased DNA oxidation. It has already been shown that mercury-induced DNA damage can be reversed in rats with bixin and norbixin (Barcelos et al., 2012). In fact, norbixin has also been shown to protect DNA from oxidative damage induced by hydrogen peroxide and metal ions in vitro (Kovary et al., 2001). Bixin has been shown to have effective singlet oxygen quenching activity (Di Mascio et al., 1990). Although the results were varied in cell response in this study, with different concentrations causing differential effects dependent on the parameter assessed, bixin clearly decreased both the oxidative and anti-inflammatory markers at all concentrations.

There is mounting evidence that bixin can be an important health supplement, particularly in terms of its potent oxygen-scavenging abilities. This study defines a number of biokinetic parameters of bixin uptake in domestic dogs; however, more extensive research would help define the most effective doses within specific time periods, which would provide support of the potential immunomodulatory and antioxidant properties of this carotenoid.

LITERATURE CITED


Bixin uptake and immunomodulation in dogs


