

Waltham International Symposium: Pet Nutrition Coming of Age

Role of Dietary Antioxidants to Protect against DNA Damage in Adult Dogs¹

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ABSTRACT We studied the effects of feeding an antioxidant blend of vitamins, minerals and carotenoids to a mixed adult dog population ($n = 40$, mean 4.4 ± 1.85 y) for a 16-wk period. Compared to the control group of dogs ($n = 20$), the antioxidant (AOX)-supplemented group of dogs ($n = 20$) demonstrated significant increases in plasma levels of vitamin E and taurine by 4 wk of supplementation ($P < 0.01$) and total antioxidant activity (as measured by ferric-reducing antioxidant power assay) by 8 wk of supplementation ($P < 0.05$). Following 8 wk of supplementation, the AOX-supplemented dogs also showed significant reductions in both endogenous and exogenous DNA damage ($P < 0.005$) compared to that of the control dogs, as measured by the comet assay. Over an 8-wk rabies vaccination course that started at 8 wk supplementation, the AOX-supplemented dogs also demonstrated significantly higher vaccine-specific virus-neutralizing antibody levels at 2, 4 and 6 wk postvaccination ($P < 0.05$) and a tendency toward establishing a vaccine-specific antibody response quicker than did the control group of dogs. These findings in dogs suggest that antioxidant supplementation can achieve sustained increases in circulating levels of antioxidants that exert a protective effect by a decrease in DNA damage, leading to improved immunological performance. These findings also have implications in a wider context where free-radical damage has been associated with a variety of degenerative disorders and the aging process in general. *J. Nutr.* 132: 1720S–1724S, 2002.

KEY WORDS: • dogs • antioxidants • DNA damage • comet assay • vaccination

Oxidation and production of free radicals, such as reactive oxygen-containing species (ROS³), are an integral part of life and the body's metabolism to the extent where they may be deliberately produced to serve important biological functions (1). However, free radicals are useful only when they are produced in the right amount at the right place at the right time. Alterations to any of these parameters leading to free-radical imbalance can lead to lipid peroxidation, cell death and genetic damage as a result of the extremely reactive nature of free radicals (2). Increasing experimental, clinical and epidemiological evidence shows an involvement of free radicals and ROS in the development of a variety of diseases including cancer and arthritis, and in the aging process (3,4).

A variety of defense mechanisms do exist to quench potentially damaging free radicals, including enzymes, micronu-

trients and excision and repair processes that remove free-radical-induced damage. These defensive measures function as part of a complex system with significant interdependence and additive or synergistic effects. As antioxidant defenses are produced within the body and/or derived from the diet, efficient functioning of the antioxidant defense system is very much dependent on the optimal functioning of the body's metabolism and nutrition. However, despite these defense systems, damage still occurs within the cell, and it is thought accumulation of unrepaired DNA may contribute to a variety of disorders associated with the aging process.

Numerous epidemiological studies to date highlight the importance of consuming dietary products rich in antioxidants (5–7). Recent studies in humans have shown that supplementation with antioxidant compounds such as vitamins E and C, lycopene and β -carotene can help reduce levels of free-radical damage (8–10). This lends support to the hypothesis that dietary products high in antioxidants potentially exert a protective effect against degenerative disorders, such as cancer, by a decrease in DNA damage (11).

Studies to understand the mutual interactions of the numerous dietary antioxidants present in foods will be important to identify supplements that have a protective effect on health and help minimize DNA damage. Determining optimal antioxidant requirements will also be essential to reduce free-radical damage, but not induce formation of prooxidants that could enhance any deleterious effects. Maintaining a balanced

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³ Abbreviations used: AOX, antioxidant; FAVN, fluorescent antibody virus neutralization test; FRAP, ferric-reducing antioxidant power; OIE, Office International des Epizooties; PBSa, phosphate-buffered saline; ROS, reactive oxygen species.

antioxidant status and reducing levels of DNA damage have become a main focus of nutritional research. One of the requirements to achieve these objectives is the ability to be able to accurately measure levels of free-radical damage and how dietary intervention may be able to reduce such damaging effects. In this report, we describe the results of a 16-wk supplementation trial in adult dogs with an antioxidant blend of vitamins, minerals and carotenoids. The effects of the antioxidant blend on antioxidant status (vitamin E and taurine), total plasma antioxidant activity [as measured by the ferric-reducing antioxidant power (FRAP) assay], DNA damage (comet assay) and *in vivo* immunological function (specific vaccination response) were evaluated.

MATERIALS AND METHODS

Animals

Two groups of 20, age- (mean 4.4 ± 1.85 y) and sex-matched adult dogs of mixed breed were chosen for the study. All dogs had been vaccinated (canine distemper virus, parvovirus and adenovirus) and were deemed clinically healthy. All dogs were housed at the Waltham Centre for Pet Nutrition (Leicestershire, UK), where the dogs were housed in purpose-built, environmentally enriched facilities (12) and treated in accordance with the Centre's research ethics and UK Home Office regulations.

Study design

All dogs were offered a base diet that was nutritionally complete and balanced, consisting of wet (Pedigree®; Masterfoods, Melton Mowbray, UK) and dry (Chappie® Complete; Masterfoods, Peterborough, UK) manufactured diets in a 50:50 ratio on an energy basis. The base diet was offered for 12 wk before commencement of the study at an allowance of 460 kJ predicted metabolizable energy per kg BW^{0.75}, designed to maintain normal body weight. The control group remained on the base diet for the 16-wk test phase, whereas the antioxidant (AOX)-supplemented group simultaneously received the base diet and were orally supplemented with the antioxidant blend (vitamin C, vitamin E, taurine, lutein, lycopene and β -carotene) on a daily basis for the 16-wk test phase. Dietary intakes were altered accordingly to account for any changes in body weight.

For analysis of plasma vitamin E, taurine and total plasma antioxidant activity, samples were collected at wk 0, 4, 8 and 12. For DNA damage, samples were collected at wk 0 and wk 8. The vaccination regimen utilizing the inactivated rabies vaccine (Nobivac® Rabies; Intervet UK, Cambridge, UK) was implemented 8 wk into the test-phase period. Blood samples were collected at 0, 1, 2, 4, 6, and 8 wk postvaccination.

Blood samples

For analysis of vitamin E, taurine and total plasma antioxidant activity, blood samples were collected from fasted dogs at wk 0, 4, 8 and 12, into foil-wrapped lithium heparin tubes (LIP, Shipley, UK) and further prepared on ice under subdued light. Plasma samples for vitamin E and taurine analysis were stored at less than -80°C until analysis. Plasma vitamin E was analyzed by the HPLC method of Hoehler et al. (13), whereas plasma taurine levels were analyzed using the method of Chiang (14). Total plasma antioxidant activity as measured by the FRAP assay was performed on an automated Cobas Fara centrifugal analyzer (Roche Diagnostics, Basel, Switzerland) according to the method described by Benzie and Strain (15).

For analysis of DNA damage, blood samples were collected at wk 0 and wk 8 into lithium heparin tubes (LIP) and diluted 1:1 in phosphate-buffered saline (PBSa). Leukocytes were isolated over His-topaque 1083 gradients (Sigma Chemical, Poole, UK) by centrifugation at $1000 \times g$ for 40 min. Leukocytes were washed twice in 10 mL PBSa and centrifuged at $700 \times g$ for 10 min before counting and freezing slowly at 1×10^6 cells/mL in 90% fetal calf serum (Sigma) and 10% dimethyl sulfoxide (Sigma) to less than -80°C until re-

quired. Viability (assessed by trypan blue exclusion) was typically around 98%. DNA damage, measured by the comet assay, was conducted according to Singh et al. (16) with slight modifications by Heaton et al. (17). DNA strand breaks were analyzed in untreated and H₂O₂-treated isolated canine leukocytes. Comets were scored based on a validated visual scoring system (100 cells per sample) using image analysis software (KOMET 4.0 analysis package; Kinetic Imaging, Liverpool, UK) and the methods of Collins et al. (18,19).

For analysis of vaccine-specific virus-neutralizing antibody, blood samples were collected into anti-coagulant-free tubes (LIP). Clotted blood samples were centrifuged at $2200 \times g$ for 10 min and the serum was removed and stored at less than -20°C until analysis. Testing for vaccine-specific virus-neutralizing antibody was carried out by the Rabies Research and Diagnostic Unit, Veterinary Laboratories Agency (Weybridge, UK) using the fluorescent antibody virus neutralization (FAVN) test according to the method of Cliquet et al. (20).

Statistical analysis

The data were evaluated using the SPSS for Windows (Version 10.0.0, SPSS Chicago, IL) using repeated-measures ANOVA to analyze the antioxidant status and vaccination data. When differences between groups were indicated by a significant time \times group interaction in the ANOVA, these were investigated in more detail by performing *t*-tests at each time point individually. Paired and unpaired *t*-tests were used to analyze DNA damage data and the time taken to reach the 0.5 IU/mL protection level for the vaccination data. All variables were assessed for normality before analysis. Values were considered significant at $P < 0.05$. Data are reported as means \pm SEM.

RESULTS

Antioxidant status

Plasma vitamin E levels were significantly increased ($P < 0.01$) in the AOX-supplemented group of dogs at the first sampling time point after 4 wk of supplementation, which was maintained for the remainder of the study period (Fig. 1). Plasma taurine levels were also significantly higher at the first sampling time point after 4 wk of supplementation ($P < 0.01$) in the AOX-supplemented group of dogs after supplementation, which was maintained for the remainder of the study period (Fig. 2). Total plasma antioxidant activity was significantly increased ($P < 0.05$) in the AOX-supplemented group of dogs after 8 wk of supplementation and was maintained for the remainder of the study period. At the first sampling time

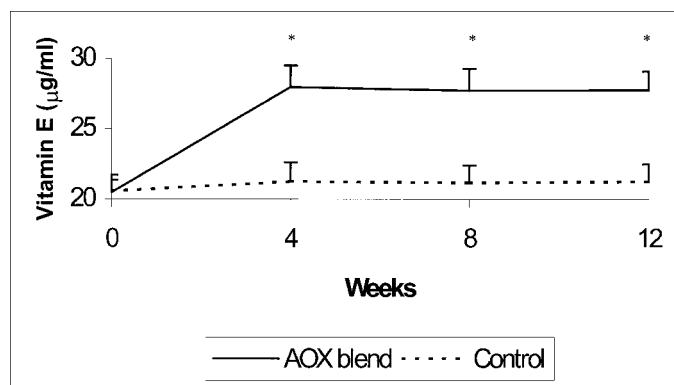


FIGURE 1 Change in plasma vitamin E levels between supplemented and nonsupplemented groups following supplementation with the antioxidant blend. Results are expressed as means \pm SEM, $n = 20$. Asterisks denote significant increases ($P < 0.01$) following supplementation with the antioxidant blend.

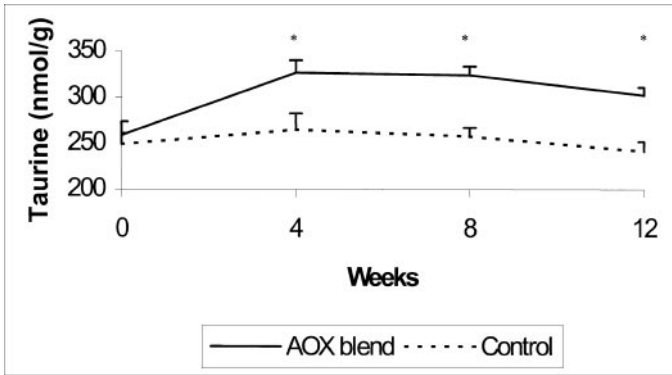


FIGURE 2 Change in plasma taurine levels between supplemented and nonsupplemented groups following supplementation with the antioxidant blend. Results are expressed as means \pm SEM, $n = 20$. Asterisks denote significant increases ($P < 0.01$) following supplementation with the antioxidant blend.

point after 4 wk of supplementation there was a tendency toward an increase in total plasma antioxidant activity, although this was nonsignificant ($P = 0.12$) (Fig. 3). No significant changes in plasma levels of vitamin E, taurine or total plasma antioxidant potential were observed in the control group of dogs at any point during the study period.

DNA damage

No significant differences were noted in endogenous or exogenous DNA damage levels between the two groups at wk 0 (Fig. 4). After 8 wk of supplementation there was a significant reduction in levels of both endogenous ($P < 0.005$) and exogenous ($P < 0.005$) DNA damage in the AOX-supplemented group of dogs, compared to those of the control group of dogs (Fig. 5). The control group of dogs showed no significant change in either endogenous or exogenous levels of DNA damage when comparing samples taken after 8 wk of supplementation to baseline levels (data not shown). However, after 8 wk of supplementation, when levels of exogenous and endogenous DNA damage from the AOX-supplemented group of dogs were compared to their own baseline values, significant reductions in endogenous DNA damage ($P < 0.05$;

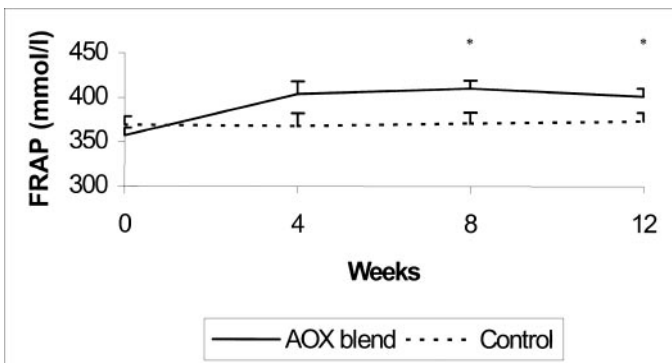


FIGURE 3 Change in total plasma antioxidant activity (FRAP) levels between supplemented and nonsupplemented groups following supplementation with the antioxidant blend. Results are expressed as means \pm SEM, $n = 20$. Asterisks denote significant increases ($P < 0.05$) following supplementation with the antioxidant blend.

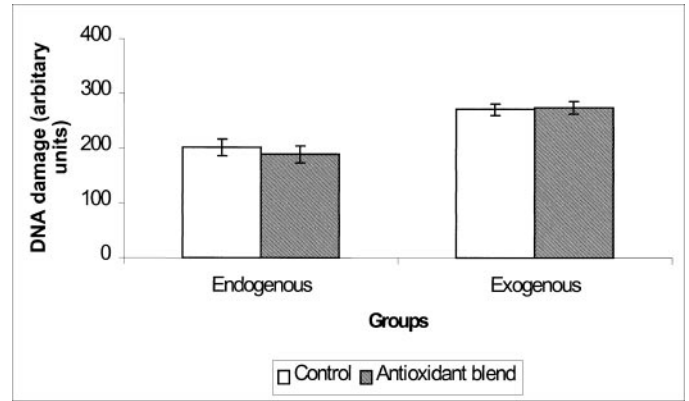


FIGURE 4 Endogenous and exogenous DNA damage in both the control and antioxidant (AOX)-supplemented groups of dogs taken presupplementation. Mean values from each group are shown. Results are expressed as means \pm SEM, $n = 20$. No significant differences were noted.

data not shown) and exogenous DNA damage ($P < 0.005$; data not shown) were observed.

Vaccination study

Production of rabies-specific neutralizing antibodies was significantly higher ($P < 0.05$) at 2, 4, and 6 wk postvaccination in the AOX-supplemented group of dogs compared to that of the control group of dogs (Fig. 6). Also, the time taken to reach a level of 0.5 IU/mL, a titer considered by the Office International des Epizooties (OIE) (21) as being the minimum protective antibody level, was determined for the two groups of dogs. Figure 6 demonstrates that, although not significant ($P = 0.18$), there was a tendency for the AOX-supplemented group of dogs to reach this protective level quicker than the control group of dogs.

DISCUSSION

It is widely believed that free radicals (e.g., superoxide and hydroxyl reactive species) are constantly generated in vivo and are significant contributors to the development of chronic degenerative disorders, such as cancer and arthritis (22). Al-

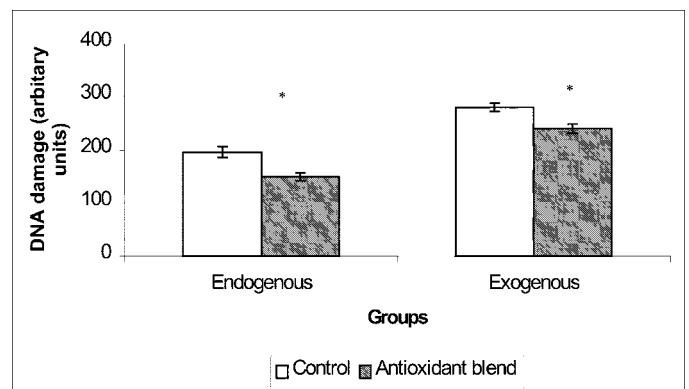


FIGURE 5 Endogenous and exogenous DNA damage in both the control and antioxidant (AOX)-supplemented groups of dogs taken at 2 mo postsupplementation. Mean values from each group are shown. Results are expressed as means \pm SEM, $n = 20$. Asterisks denote significance of $P < 0.005$.

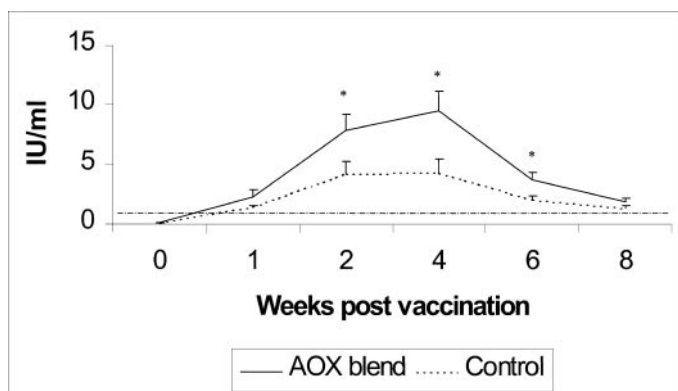


FIGURE 6 Comparison of vaccine responses to rabies vaccination between the control and antioxidant (AOX)-supplemented groups of dogs. Results are expressed as means \pm SEM, $n = 20$. Asterisks denote significant increases ($P < 0.05$) following supplementation between supplemented and nonsupplemented groups with the antioxidant blend. The dashed line represents the OIE protective antibody level of 0.5 IU/mL.

though multiple antioxidant repair systems exist to help control this process, reactive species still cause damage to biomolecules, cells and tissues in a variety of ways. Damage to DNA is probably the most significant biological target because this may ultimately lead to altered gene expression, disruption of cellular repair mechanisms and distorted cellular function. It is thought that continual damage to DNA is a significant contributor to many age-related pathological diseases (22).

Studies in humans indicate that consuming food products high in antioxidants, or antioxidant supplements are linked with a decreased risk of cancer (5), coronary heart disease (6) or enhanced and maintained immunological performance (23). Antioxidants commonly associated with these beneficial effects include vitamins E and C, and carotenoids such as β -carotene. However, conflicting data have been reported from intervention studies utilizing some of these components. Whereas Meydani et al. (24) demonstrated beneficial immunological effects following vitamin E supplementation in elderly subjects, Harman and Miller (25) demonstrated no such effects. The concept of β -carotene being a potent anti-cancer agent was challenged recently when supplementation trials with β -carotene alone in humans actually demonstrated an increased incidence of certain cancers in high-risk populations (26,27).

Inevitably, the variability associated with study design, data analysis and the difficulty in accurately controlling and recording food intakes may contribute to such dramatic differences, and highlights some of the inherent problems associated with human supplementation studies (8,28–30). From a companion animal perspective, there are significant advantages in being able to carefully monitor and control dietary and antioxidant supplement intake, thus helping to keep such variables to a minimum.

Results from the present study indicate that supplementation of a mixed adult dog population with a blend of antioxidants significantly increases plasma levels of vitamin E and taurine, which was also associated with a significant increase in total plasma antioxidant activity. In addition, the increases in plasma vitamin E, taurine and antioxidant activity were associated with a significant reduction in both endogenous and exogenous levels of DNA damage and an improved immune response to vaccination.

The significantly increased levels of plasma vitamin E and

taurine in conjunction with increased total plasma antioxidant activity (measured by FRAP assay) following consumption of a blend of antioxidants support previous studies we have conducted that have demonstrated significant increases in plasma nutrient levels and antioxidant potential in cats (31,32) and dogs (R. Obra, unpublished observations, 1998), and indicate a direct absorption of specific antioxidants that potentially enhance the ability to sequester damaging free radicals. It is important to emphasize that, given the complex interactions and synergisms of the antioxidant defense system, total antioxidant activity of plasma should always be considered in relation to individual plasma antioxidants to give an accurate indication of antioxidant status (33).

Although significant increases in plasma levels of vitamin E, taurine and total antioxidant activity may increase the protective effects against free-radical damage, it is important to show that these increases have a functional impact. Measuring DNA damage using the comet assay is one method by which this can be achieved and has been used in a variety of studies to assess the potentially beneficial effects of dietary intervention on free-radical damage (8,9,34,35).

The present data demonstrate that at wk 0 (presupplementation) there were no differences in DNA damage between the two groups of dogs, but after 8 wk of supplementation there was a significant reduction in both endogenous and exogenous DNA damage. A reduction in endogenous damage can indicate increased protection of DNA by antioxidants in the supplement against free-radical attack and/or increased rates of repair to damaged DNA. Challenging leukocytes in vitro with exogenous H_2O_2 to induce DNA strand breaks also provides an indication of antioxidant protection or resistance to free-radical damage. As highlighted by Duthie et al. (8), human subjects who were supplemented daily with vitamin C, vitamin E and β -carotene showed increased resistance to in vitro H_2O_2 -induced DNA damage compared with cells from subjects who received a placebo, suggesting antioxidant supplementation increases cellular resistance to free-radical damage.

Decreases in DNA damage may indicate potential protection against oxidative stress involved in various diseases (19). Similar reductions in exogenous DNA damage (>25%) were observed in previous studies where the suggestion was made that suppressing free-radical damage could potentially reduce DNA instability, mutation and dysfunction, thus supporting the hypothesis that antioxidants exert a protective effect against degenerative disorders (e.g., cancer) by a decrease in DNA damage (8,9).

Although differing results have been obtained between human studies with regard to antioxidant supplementation and boosting vaccinal responses (24,25), the present study clearly shows a relationship between a significant increase in vaccine-specific antibody titer and antioxidant supplementation in adult dogs. Recent evidence suggests that accumulation of DNA damage may have a detrimental impact on the effectiveness of the immune system to respond to infectious pathogens, particularly in elderly subjects (10,36). Although age was not a factor under investigation in the present study, it is clear from our results and from the emerging consensus that DNA damage can potentially impair immune function that the significant reductions in both endogenous and exogenous DNA damage observed seem to have contributed to the significantly improved immune performance of the AOX-supplemented group of dogs.

Because this study made use of a cocktail of antioxidants, it is not possible to attribute these protective effects to an individual component. Other parameters indicative of antioxidative status and oxidative stress in the blood have also been

measured together with biomarkers of bioavailability, molecular interactions and immune function (P. Heaton, unpublished results, 2002). The current findings, however, support the hypothesis that antioxidant supplementation can achieve sustained increases in circulating levels of antioxidants that exert a protective effect by a decrease in DNA damage, leading to improved immunological performance. These findings also have implications in a wider context where free-radical damage has been associated with a variety of degenerative disorders, such as cancer (37), arthritis (38), neurodegenerative disease (39) and cardiovascular disease (6).

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