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Chicken Model for Studying Dietary Antioxidants Reveals that Apple (Cox's Orange)/Broccoli (*Brassica oleracea* L. *var. italica*) Stabilizes Erythrocytes and Reduces Oxidation of Insoluble Muscle Proteins and Lipids in Cooked Liver

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A chicken model for studying the effects of antioxidants in the diet on oxidative status was set up. Chickens fed a semi-synthetic diet low in antioxidants showed a remarkable decrease in erythrocyte stability toward H_2O_2 or 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), but increases in catalase activity in liver, carbonyls in insoluble muscle proteins, and enhanced lipid oxidation in heat-treated liver samples compared to that of conventionally fed chickens. Thus, this chicken model proved to be more susceptible to oxidative changes than conventionally fed chickens, reflecting a low antioxidative defense. Supplementing this low antioxidant diet with 10% apple/broccoli mixture counteracted these changes, except for activity of catalase in the liver and AAPH-induced lysis of erythrocytes. Supplementation with 10% sweet corn only reduced the carbonyl content in insoluble proteins. However, neither low antioxidant diet nor vegetable supplements affected selected antioxidative enzymes or oxidative stability of lipids in heat-treated muscle tissue.

KEYWORDS: Chicken; apple; broccoli; sweet corn; oxidative stress; meat

INTRODUCTION

Epidemiological studies indicate a number of beneficial health effects of increased intake of fruit and vegetables (*1*, *2*), which are rich sources of antioxidants. The role of dietary antioxidants such as vitamins C and E have been studied extensively (*3*−*5*) in both animal and human intervention studies focusing on improving the antioxidative status and, as for animal studies, oxidative stability of meat. Other antioxidants than vitamins C and E are also abundant in fruit and vegetables, e.g., polyphenols (*6*, *7*) and carotenoids (*8*, *9*). These compounds, not essential vitamins in themselves, may contribute directly or indirectly to lower the endogenous oxidative stress (*10*). Intestinal absorption and subsequent tissue deposition of antioxidants from the diet are necessary for a direct effect, but dietary antioxidants may also have a local effect within the gastrointestinal tract, e.g., as free radical scavengers or iron chelators (*11*). Both the antioxidative defense and oxidative stability of meat may thus improve with increasing intake of fruit and vegetables due to naturally occurring antioxidative compound(s).

The primary aim of the present work was to set up an animal model for studying the effects of antioxidants/antioxidant-rich diet on selected markers of antioxidative status and second to study oxidative stability in muscle and liver. Chickens were fed a semi-synthetic diet without added vitamin E and butylated hydroxytoluene (BHT) (low antioxidant diet). We studied the effect of supplementing this low antioxidant diet with sweet corn or a mixture of apple and broccoli. Vegetables were supplemented only on a 10% energy basis to avoid possible decreases in weight gain compared to the control.

MATERIALS AND METHODS

Animals and Study Design. Female chickens (Ross 208) were obtained from a commercial hatchery as 1 day olds, and raised at our local facility under standard conditions, with free access to water and feed. Chickens were housed in pens (20 chickens per 1.44 m^2) and fed a semi-synthetic diet including BHT and synthetic vitamin E up to day 21. After 21 days, chickens were fed a semi-synthetic diet without BHT and synthetic vitamin E (low antioxidant diet) as described in **Table 1**. This semi-synthetic diet consisted of isolated soybean protein, glucose from hydrolyzed starch, and refined vegetable oil. The main feature of this diet is its low content of natural antioxidants, primarily tocopherols and polyphenols, particularly those associated with the dietary fiber fraction. By designing a diet without known antioxidants, it should be possible to study the effect of antioxidants naturally occurring in the vegetables, which may not, on an individual basis be significant, but in combination act synergistically. From day 21, chickens were fed either sweet corn (*Zea mays* L. *var. Saccharata*), a mixture of apple (Cox's Orange) and broccoli (*Brassica oleracea* L. *var. italica*) (1:1 wt/wt) constituting approximately 10% based on energy, or continued

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Table 1. Composition of Semi-Synthetic and Conventional Diets[a]

ingredients	conventional (%)	semi-synthetic (%)
wheat	47.5	
soybean cake	22.1	
peas	10	
rapeseed cake	3.8	
rapeseed oil	2.3	
soybean oil	3.0	
animal fat	2.0	
fish meal	3.0	
isolated soybean protein		25
glucose		60
cellulose		3.0
refined corn oil		2.0
refined rapeseed oil		2.0
glycine		0.4
DL-methionine	0.2	0.6
vitamin mixture	1.2	1.2[b]
mineral mixture	5.0	5.7

[a] Low antioxidant diet is defined as semi-synthetic diet without added vitamin E and butylated hydroxytoluene (BHT). Vitamin and mineral content per kg diet: retinol 4500 IU; cholecalciferol 450 ICU; α-tocopherol 50 IU; menadione 1.5 mg; biotin 0.6 mg; cyanocobalamin 20 μg; choline chloride 2 g; folic acid 6 mg; niacin 50 mg; pyridoxine·HCl 7.8 mg; riboflavin 15 mg; thiamin·HCl 15 mg; BHT 100 mg; $CaHPO_4 \cdot 2H_2O$ 20.7 g; $CaCO_3$ 14.8 g; KH_2PO_4 10.0 g; NaCl 6.0 g; $MgSO_4 \cdot 7H_2O$ 6.0 g; KCl 1.0 g; $MnSO_4 \cdot H_2O$ 350 mg; $ZnCO_3$ 150 mg; $Fe_2(SO_4)_3$ 500 mg; $CuSO_4 \cdot 5H_2O$ 30 mg; Na_2SeO_3 0.2 mg; KJO_3 2 mg; $CoCl_2$ 1.7 mg; $Na_2MoO_4 \cdot 2H_2O$ 8.3 mg. [b] α-Tocopherol and BHT were not included in the low antioxidant diet after day 21.

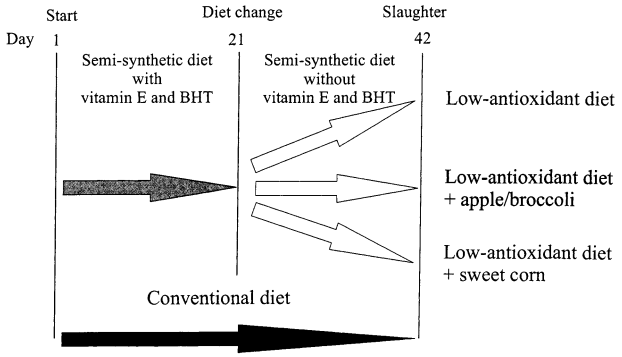

Figure 1. Feeding regimen of the chicken diet from day 1 to slaughter at day 42.

on semi-synthetic diet until slaughter, at 42 days. Chickens ate the supplements before they were given free access to the low antioxidant diet. A control group received a conventional diet (Table 1) throughout the experimental period. Chickens were weighed and blood drawn at 42 days, i.e., after 21 days on experimental diet. A feeding regimen of the chicken diet is given in **Figure 1**.

Erythrocyte Stability. Erythrocyte stability was determined by a micro-method (*12*). Briefly, blood was drawn from the brachial vein (cutaneous ulnar vein) and mixed with EDTA. Suspensions of 0.6−0.8% (vol/vol) erythrocytes in PBS was mixed 1:1 with PBS containing H_2O_2 or 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) (PolySciences, Warrington, PA) followed by a control with cells lysed by addition of Triton X-100 (1%). This control allowed correction for the amount of erythrocytes in the blood sample, as well as for the effect of oxidant on absorbance of hemoglobin. Samples were incubated shaking for 20 h, residual cells pelleted, and cell lysis determined by measuring the content of hemoglobin in the supernatant at 406 nm.

Antioxidative Enzymes. Antioxidative enzymes were determined in M. pectoralis major (PM), M. iliotibialis (IL), and liver. One hundred milligrams of frozen tissue was homogenized in 0.8 mL of homogenization buffer (0.01 M Tris-HCl, pH 7.4, 1 mM EDTA, 0.25 M sucrose) on ice with an Ultra-Turrax (T25, IKA-Labortechnik, Staufen, Germany) for 5 s at 13 500 rpm. The homogenate was centrifuged at 21000*g* for 30 min at 4 °C, and the supernatant was stored at −80 °C until analysis. The activities of antioxidative enzymes were assayed as described: catalase (CAT) (*13*), glutathion peroxidase (Gpx) (*14*), and superoxide dismutase (SOD) (*15*), except that microtiter plates were used. All samples were measured in triplicate at appropriate dilutions to give activities of the enzymes in the linear range of standard curves constructed with pure enzymes (Sigma, St. Louis, MO). Protein content of homogenates were determined using the bicinchoninic acid assay (Pierce, Rockford, IL) and bovine serum albumin as standard.

Lipid Oxidation. Lipid oxidation was induced by heating whole liver (∼10 g) or meat patties from minced PM or IL muscle (5 g) in polypropylene plastic bags in a water bath at 70 °C for 10 min, and then allowed to develop through storage for 1, 3, or 6 days at 4 °C in oxygen-permeable polyethylene zip-lock bags. Lipid oxidation products were then determined by a micro-method for thiobarbituric acid reactive substances (TBARS). Samples of ∼100 mg of the meat patties or liver were incubated in 0.5 mL of 50% trichloroacetic acid containing 1.3% (w/v) thiobarbituric acid and heated to 60 °C for 1 h, followed by determination of absorbance at 532 nm. Tetraethoxypropane (Sigma, St. Louis, MO), which spontaneously decomposes in an aqueous environment to form malondialdehyde (MDA), was used as a standard and absorbance expressed as MDA equivalents.

Protein Oxidation. Protein oxidation was determined by the dinitrophenylhydrazine (DNPH) method as described (*16*) using total PM homogenates (gross carbonyl content). Briefly, frozen tissue (0.5 g) was homogenized in 2 mL of homogenization buffer (10 mM TRIS, pH 7.4, 250 mM sucrose, 1 mM EDTA) using an Ultra-Turrax (T25, IKA-Labortechnik, Staufen, Germany) for 2 × 10 s at 12 000 rpm in ice water with intermittent cooling for 20 s. Samples (50 μL) of total (prior to centrifugation) or soluble protein in supernatant obtained by centrifugation at 21000*g* for 30 min were incubated with 200 μL of 20% trichloroacetic acid (TCA) in a microfilter plate (MultiScreen FC plate, Millipore, Bedford, MA). Soluble material was aspirated by applied suction, and the precipitate was washed twice in 200 μL of 20% TCA, and then incubated with 200 μL of 2 N HCl containing DNPH at 2 mg/mL for 5 min at room temperature; unreacted DNPH was aspirated, the precipitate washed five times with ethanol/ethyl acetate (1:1), and the sample was redissolved in 6 M guanidinium overnight at room temperature, and filtered into a microtiter plate for determination of absorbance at 357 nm.

Tocopherol Analysis. Tocopherols were determined by normal phase HPLC after saponification (*17*).

Statistical Analyses. Data were analyzed using the GLM procedure of SAS, version 8.01 (SAS Inst. Inc., Cary, NC). The statistical model included the fixed effects of diet and tissue. Results are presented as means ± SEM of 20 chickens, unless otherwise stated.

RESULTS

Chicken Weight. Conventionally fed chickens were larger (1702 ± 58 g) than those fed the low antioxidant diet (1363 ± 28 g). There was no effect of supplementing the low antioxidant diet with apple/broccoli (1412 ± 42 g) or sweet corn (1379 ± 36 g).

Vitamin E Content in Diet, Plasma, Erythrocytes, and Tissue. The α-tocopherol content in the conventional chicken diet was 65.8 ± 4.9 μg/g and below the detection limit of 0.1 μg/g in the low antioxidant diet. Chickens fed a low antioxidant diet, had approximately 10 times less α-tocopherol in plasma (0.89 ± 0.17 vs 10.9 ± 0.5 μg/g) and erythrocytes (0.05 ± 0.01 μg/g vs 0.86 ± 0.07 μg/g) compared to that of chickens on a conventional diet. Preliminary studies showed that addition of apple/broccoli to either diet did not alter the levels of α-tocopherol in plasma (0.52 ± 0.13 μg/g) or erythrocytes (0.07 ± 0.01 μg/g). The α-tocopherol content in PM of conventionally fed chickens (7.96 ± 0.61 μg/g) was higher than that of chickens on the low antioxidant diet (2.35 ± 0.18 μg/g), and neither apple/broccoli (2.39 ± 0.10 μg/g) nor sweet corn (2.46 ± 0.11 μg/g) supplements to the low antioxidant diet changed the levels of α-tocopherol in PM.

Table 2. Activities (U/g of wet tissue) of the Antioxidative Enzymes Catalase (CAT), Glutathion Peroxidase (Gpx), and Superoxide Dismutase (SOD) in M. Pectoralis Major, M. Iliotibialis, and Liver from Eight Chickens (mean (SEM))

	M. pectoralis major			*M. iliotibialis*			liver		
diet	CAT	Gpx	SOD	CAT	Gpx	SOD	CAT	Gpx	SOD
low antioxidant	6.8 (0.6)	1.41 (0.2)	602 (14)	12.1 (1.7)	1.05 (0.1)	414 (19)	541 (28)[a]	27.1 (1.9)	2526 (122)
low antioxidant + apple/broccoli	6.1 (0.8)	1.23 (0.2)	583 (42)	13.3 (2.0)	0.98 (0.1)	504 (36)	644 (58)[a]	27.7 (2.2)	2641 (83)
low antioxidant + sweet corn	6.6 (0.6)	1.09 (0.1)	613 (17)	11.2 (1.3)	0.87 (0.1)	462 (24)	513 (47)	23.1 (0.9)	2410 (125)
conventional	6.5 (0.6)	1.03 (0.1)	582 (12)	8.7 (0.8)	0.93 (0.0)	378 (19)	414 (20)	23.1 (0.9)	2718 (54)

[a] Values significantly different from conventional ($P < 0.05$).

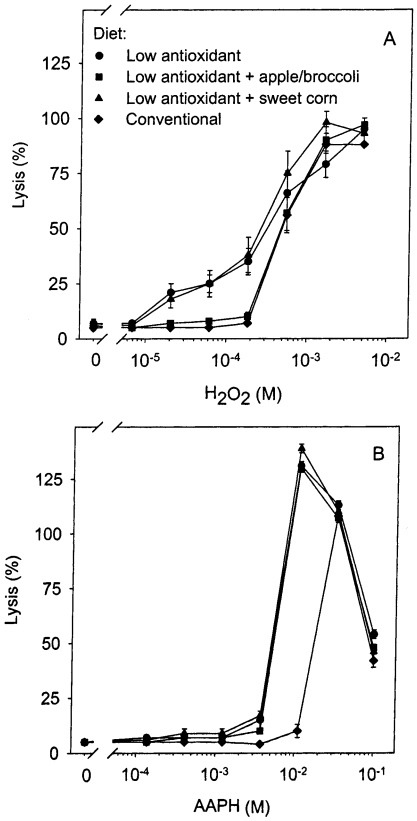

Figure 2. Stability of erythrocytes exposed to H_2O_2 (A) and 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) (B). Data (mean ± SEM) for eight chickens per treatment are presented.

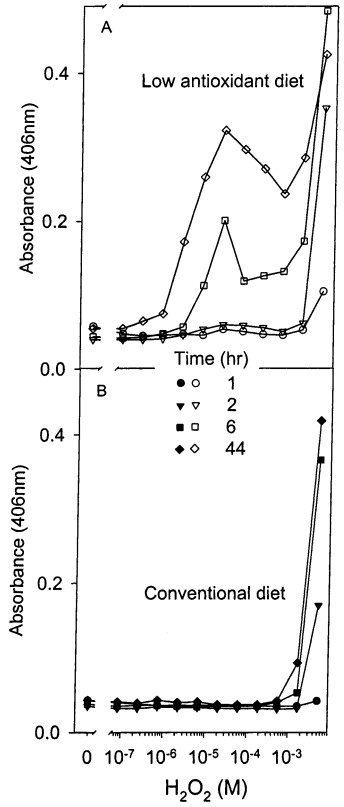

Figure 3. Stability of erythrocytes exposed to H_2O_2 for 1, 2, 6, or 44 h. Erythrocytes data from chickens fed a low antioxidant diet (A) and conventional diet (B) are presented. Data for one of two comparable experiments are shown.

Erythrocyte Stability. Both H_2O_2- and AAPH-induced lysis were approximately 3-fold higher in erythrocytes from chickens fed a low antioxidant diet compared to that of erythrocytes from chickens fed a conventional diet (**Figure 2**). In addition to this difference, erythrocytes from chickens on the low antioxidant diet displayed a biphasic stability curve when exposed to H_2O_2. Apparently, approximately one-third of the population of erythrocytes had higher sensitivity toward lysis by H_2O_2 compared to lysis of the residual erythrocytes. The presence of two erythrocyte populations in low antioxidant chickens is also illustrated in **Figure 3**A, which shows kinetics of lysis of erythrocytes when exposed to H_2O_2, forming the shoulder that is also apparent in **Figure 2A**. Interestingly, chickens on a low antioxidant diet supplemented with apple/broccoli showed the presence of only one population of erythrocytes of high resistance toward H_2O_2 and virtually similar to erythrocytes from conventionally fed chickens, whereas sweet corn had no effect. Neither sweet corn nor apple/broccoli supplement affected AAPH-induced erythrocyte lysis.

Antioxidative Enzymes. The activity of CAT was increased in liver of chickens on the low antioxidant diet with or without apple/broccoli supplementation, relative to that of conventionally fed chickens (**Table 2**). There was no significant effect of diets on either Gpx or SOD activities in any of the tissues analyzed.

Protein Oxidation. Protein oxidation determined as gross carbonyl content in insoluble proteins from PM was increased in chickens fed the low antioxidant diet compared to chicken on conventional diet, whereas carbonyl content in soluble proteins were not significantly affected. Addition of both apple/

Table 3. Mean Carbonyl Content (pmol/mg of protein) in Soluble and Insoluble Proteins from M. Pectoralis Major of Eight Chickens Per Treatment (mean ± SEM)

	carbonyl content	
diet	soluble proteins	insoluble proteins
low antioxidant	169 ± 13	98 ± 9[a]
low antioxidant + apple/broccoli	156 ± 17	65 ± 5
low antioxidant + sweet corn	169 ± 9	58 ± 3
conventional	147 ± 11	64 ± 5

[a] Values significantly different from conventional ($P < 0.05$).

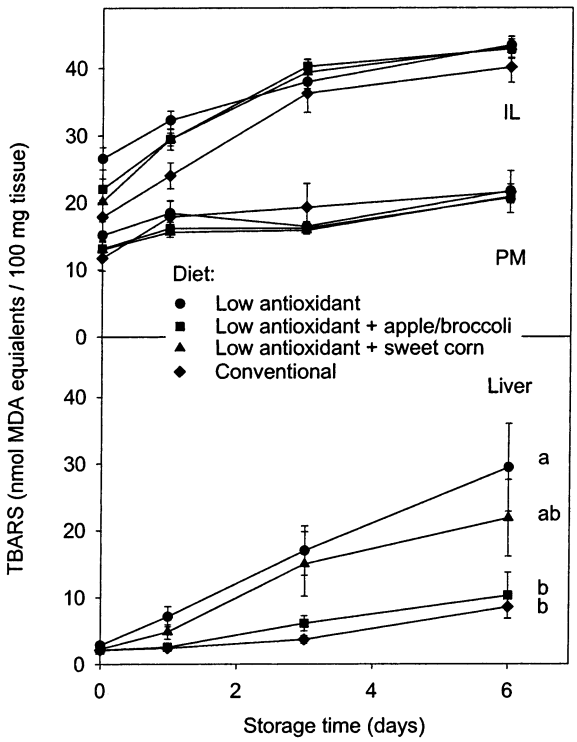

Figure 4. Thiobarbituric acid reactive substances (TBARS) expressed as malondialdehyde (MDA) equivalents/100 mg of cooked tissue. Lines marked with different letters are significantly different on day six of storage ($P < 0.05$). No differences in TBARS in M. pectoralis major (PM) or M. iliotibialis (IL). Data for eight chickens per treatment are presented (mean ± SEM).

broccoli and sweet corn to the low antioxidant diet reduced the carbonyl content in insoluble proteins to a level comparable to that of conventionally fed chickens. However, supplements did not affect carbonyl formation in soluble proteins (**Table 3**).

Lipid Oxidation. Chickens fed a low antioxidant diet had higher concentrations of TBARS in heat-treated liver after 6 days of storage compared to conventionally fed chickens, whereas the concentration of TBARS in heat-treated PM and IL was unaffected by any changes in the diet (**Figure 4**). Apple/broccoli supplemented low antioxidant diet reduced the concentration of TBARS in heat-treated liver to a level comparable to that of conventionally fed chickens, but no effect of sweet corn supplement was observed.

DISCUSSION

Increased dietary vitamin E improves oxidative status in living animals (*18*−*20*), and a drastic decrease in vitamin E has also been shown to adversely affect the redox state in chicken muscle (*21*). Exclusion of known antioxidants from the chicken diet reduced levels of vitamin E in both plasma and erythrocytes by approximately 10-fold and muscle levels by approximately 3-fold compared to that of chickens on a conventional diet. Activities of CAT in liver was increased by ∼20% following a low antioxidant diet, but there was no significant effect on activities of either Gpx or SOD; however, none of the enzymes were affected in muscle tissue in accordance with previous observations for antioxidative enzymes in turkey muscle (*22*).

Lipid oxidation in cooked liver and protein oxidation in insoluble muscle proteins from chickens on low antioxidant diet were increased compared to that of chickens on a conventional diet. This is in accordance with studies reporting reduced lipid oxidation in the liver (*23*) and slightly lower oxidation of proteins in muscle tissue of chicken after vitamin E supplementation (*22*, *24*). Lipid oxidation in minced, cooked muscle tissue was not affected by the low antioxidant diet, whereas a number of other studies find a protective effect of vitamin E supplement on lipid oxidation in muscle tissue (*22*−*25*). Removal of known antioxidants from the diet was expected to cause increased lipid oxidation in the muscle tissue. However, unpublished results from a pilot study using this low antioxidant diet showed that the content of polyunsaturated fatty acids (linoleic acid and γ-linolenic acid) in PM was approximately half that of the content in PM from conventionally fed chickens. This increased content of unsaturated fatty acids would increase the oxidative susceptibility of lipids in PM from conventionally fed chickens and seems to have counteracted a possible effect of the low antioxidant diet on lipid oxidation in muscle tissue.

Erythrocytes from chickens on low antioxidant diet were three times more susceptible to oxidative challenge by both AAPH and H_2O_2 compared to those from chickens on conventional diet. This stabilizing effect of vitamin E supplementation on erythrocytes has been reported for humans (*26*, *27*) although not to the same extent as obtained in the present study. Erythrocytes were exposed to a wide concentration range of H_2O_2, which led to the observation of a biphasic stability curve when chickens were fed a low antioxidant diet for 21 days. This may be ascribed to a mixture of relatively stable erythrocytes formed before the elimination of vitamin E and BHT from the diet and the more labile erythrocytes formed after. Approximately one-third of the population of erythrocytes had higher sensitivity toward lysis by H_2O_2 compared to lysis of the residual erythrocytes. The fraction of erythrocytes with increased sensitivity would be influenced by the rate of erythrocyte turnover and length of the antioxidant-free experimental period.

Our results thus show that erythrocyte stability, activity of CAT in the liver, lipid oxidation in cooked liver, and the concentration of carbonyls in insoluble muscle proteins are suitable parameters for assessing changes in oxidative status for this chicken model.

Chickens on a low antioxidant diet were supplemented with either apple/broccoli or sweet corn to test if the increased oxidative susceptibility could be alleviated to some extent. Apple/broccoli supplements effectively prevented the apparent formation of H_2O_2-sensitive erythrocytes, since no biphasic stability curve was observed. This indicates the presence of antioxidative compounds in apple/broccoli, possibly carotenoids or polyphenols, capable of stabilizing erythrocytes toward H_2O_2-induced lysis. Also, lipid oxidation in heat-treated liver and carbonyl formation in insoluble muscle proteins was reduced in apple/broccoli supplemented chickens. The apple/broccoli supplement was as effective as the conventional diet. The only effect we observed for sweet corn supplementation was reduced carbonyl formation in insoluble proteins.

In conclusion, we set up a chicken-model for studying effects of dietary supplements rich in antioxidants. Particularly, AAPH- and H_2O_2-induced lysis of erythrocytes increased remarkably, but also the activity of CAT in the liver, lipid oxidation in the liver, and carbonyl content in insoluble muscle proteins increased when chickens were fed a low antioxidant diet compared to a conventional diet. Apple/broccoli supplemented to a low antioxidant diet counteracted these effects, except for activity of CAT in the liver and AAPH-induced lysis of erythrocytes. However, only carbonyl content in insoluble proteins was reduced after sweet corn supplementation. Neither selected antioxidative enzymes, nor oxidative stability of lipids

in heat-treated muscle tissue were affected by the low antioxidant diet or vegetable supplements. The oxidative stability of meat was thus not decreased after the low antioxidant diet, nor was it improved after sweet corn or apple/broccoli supplementation.

The observed effects of vegetable supplementation on antioxidative parameters underlines the applicability of this oxidation-sensitive model for testing effects of dietary components on antioxidative status.

ABBREVIATIONS USED

BHT, butylated hydroxytoluene; AAPH, 2,2′-azobis (2-amidinopropane) dihydrochloride; TBARS, thiobarbituric acid reactive substances; PM, M. pectoralis major; IL, M. iliotibialis; CAT, catalase; Gpx, glutathion peroxidase; SOD, superoxide dismutase; MDA, malondialdehyde.

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