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The effect of dietary corn oil, vitamin E, and selenium on lipid peroxidation and hemorrhage in chicken liver

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The involvement of lipid peroxidation in the development of liver hemorrhages in egg-producing chickens was investigated. Two white leghorn strains of birds, a commercial layer strain and strain UCD-003, which is predisposed to the development of liver hemorrhages, were fed diets containing 5% corn oil with or without added vitamin E (11 IU/kg) and selenium (0.1 mg/kg). The extent of liver hemorrhage increased in the UCD-003 birds, particularly after they were fed the diet without added vitamin E and selenium for 3 weeks. Hepatic levels of malondialdehyde and lipid hydroperoxides increased by feeding with the experimental diets, with higher levels obtained in UCD-003 birds than in normal birds. The highest levels of malondialdehyde were found in UCD-003 birds after feeding the diet without added vitamin E and selenium for 3 weeks. Hepatic glutathione peroxidase activity decreased during the first week in normal birds and the first 2 weeks for UCD-003. The activity of catalase also tended to decrease in the first 2 weeks in the birds fed the diet without supplemental antioxidants. Superoxide dismutase activity increased dramatically in UCD-003 birds by 2 weeks, while smaller changes in activity were found in normal birds. We conclude that increased lipid peroxidation occurs in birds fed diets containing 5% corn oil along with increased liver hemorrhage in UCD-003 birds. Supplementary vitamin E and selenium decrease the amount of lipid peroxidation and lower the incidence of liver hemorrhage in UCD-003 birds fed these diets. (J. Nutr. Biochem. 8:629–633, 1997) © *Elsevier Science Inc. 1997*

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Introduction

Increased lipid peroxidation has for a long time been linked with a number of degenerative diseases[1,2] and occurs as a result of the ingestion of a number of toxins and prooxidants.[3] The involvement of lipid peroxidation in liver injury in mammalian systems has been reviewed.[4] However, the degree of pathological tissue damage that can be linked directly to lipid peroxidation in vivo is usually quite low. In contrast, egg-producing chickens can suffer from a condition known as fatty liver hemorrhagic syndrome (FLHS), which is characterized by the accumulation of large amounts of fat in the liver with the subsequent development of liver hemorrhages.[5,6] We have previously suggested that increased lipid peroxidation is responsible for the development of hemorrhagic lesions in chicken liver.[7,8]

The avian liver is a good potential model system to study lipid peroxidation, since the lipid content can be as high as 40% of the dry weight of the liver. In mammals, the major site of lipogenesis is the adipose tissue, while in birds more than 90% of fat synthesis occurs in the liver.[9] Lipids synthesized in the liver are transported to the growing oocyte as components of lipoproteins, primarily very low-density lipoprotein (VLDL), to form the egg yolk. The activity of fatty acid synthase in avian liver increases dramatically in response to increased circulating levels of estrogen as the female reaches sexual maturity. In some conditions, such as excess consumption of high energy diets or insufficient production of lipoproteins, excess lipid accumulates in the liver of birds in active egg production.[7] Some genetic lines of birds, such as the UCD-003 strain, have increased hepatic lipid levels and are more predisposed to the development of liver hemorrhages.[8,10]

The extent of lipid peroxidation that has occurred can be

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assessed by a number of different methods, including measurements of malondialdehyde (MDA) and lipid hydroperoxide levels in tissues. As well, the activities of the antioxidant defense enzymes glutathione peroxidase (GSHPx), superoxide dismutase (SOD), and catalase can be altered in response to oxidative stress. The use of a number of different parameters is necessary to assess the degree of lipid peroxidation that has occurred in vivo. Thus, the accumulation of MDA and lipid hydroperoxides in tissues can be affected not only by the production of these compounds, but also by the rate of metabolism and clearance. The activities of the different antioxidant defense enzymes do not always change in response to oxidative stress.

High levels of polyunsaturated fatty acids (PUFA) in the diet results in decreased levels of vitamin E in the body[11] and increased lipid peroxidation in vivo.[11,12] Vitamin E breaks free radical chain reactions and thus inhibits lipid peroxidation to result in decreased formation of MDA[13] and consequently may reduce the incidence of FLHS.[14] Selenium is an essential component of GSHPx, and selenium deficiency can result in decreased GSHPx activity and lead to an increase susceptibility to oxidative injury.[15] GSHPx activity was increased by feeding diets containing menhaden oil.[11]

We have previously shown that the extent of hepatic lipid peroxidation induced by injection of iron nitrilotriacetate is more severe in UCD-003 birds than in normal-laying hens.[8] This suggests that there is a link between the development of liver hemorrhages and lipid peroxidation in avian liver. In this study, the effect of feeding diets supplemented with corn oil, either with or without added vitamin E and selenium, on the extent of lipid peroxidation and liver hemorrhage in normal and in strain UCD-003 chickens was investigated. The extent of lipid peroxidation was assessed by measuring the levels of MDA, lipid hydroperoxides, SOD, GSHPx, and catalase in the liver at weekly intervals for 4 weeks.

Materials and methods

Reagents

2-Thiobarbituric acid, butylated hydroxytoluene, reduced glutathione, glutathione reductase (type IV, from yeast), *t*-butyl hydroperoxide, cumene hydroperoxide, xanthine (grade V), ferricytochrome C (type III, from horse heart), and xanthine oxidase (grade III, from buttermilk) were purchased from Sigma Chemical Company (St. Louis MO). MDA bisdimethyl acetal was obtained from Aldrich Chemical Company (Milwaukee, WI). Trichloroacetic acid (ACS grade), acetonitrile (high performance liquid chromatography [HPLC] grade) and tetrahydrofuran (HPLC grade) were purchased from Fisher Chemical Company (Mississauga, Ontario). All other chemicals used were of analytical grade or equivalent.

Animals and diets

Two strains of single-comb white leghorn hens, a normal commercial strain and strain UCD-003, which is susceptible to fatty liver rupture,[10] were used for each of two dietary treatments. Corn/soya diets containing 5% corn oil were used with either vitamin E (11 IU/kg) and selenium (0.1 mg/kg) (diet 1) or without added vitamin and selenium (diet 2), as shown in *Table 1*. Feed intake was measured weekly, and egg production was recorded daily over 4-week period. The birds were maintained on a 14 hr light to 10 hr dark cycle and were in active egg production. Twenty-four individually caged birds were used for each dietary treatment and strain in a 2 × 2 factorial design, with six birds of each strain sampled at the beginning of the experiment for a total of 108 birds. Six birds per group were killed at weekly intervals, and samples of blood and liver were taken for analysis. The degree of hemorrhage in the liver was visually scored using a five-point scale[6] as follows: 1 = no hemorrhages, 2 = one to five hemorrhages, 3 = six to 15 hemorrhages, 4 = 16 to 25 hemorrhages, 5 = more than 25 hemorrhages or massive hemorrhage of the liver. Hemorrhages were counted on both the dorsal and ventral surfaces of the liver.

Table 1 Composition of diets

Composition (g/100g)	Diet 1	Diet 2
Corn	59.35	59.35
Soybean meal (48% CP)	25.0	25.0
Calcium phosphate (20% P)	1.5	1.5
Limestone	8.0	8.0
Corn oil	5.0	5.0
Iodized salt (0.015% KI)	0.3	0.3
DL-methionine	0.1	0.1
Vitamin-mineral premix 1*	0.75	—
Vitamin-mineral premix 2†	—	0.75
Calculated nutrient content		
Metabolizable energy (kcal/kg)	3002	
Crude protein	17.2%	
Crude fat	7.3%	

*Supplied per kilogram of diet: vitamin A, 800 IU; vitamin D, 1600 IU; vitamin E, 11 IU; riboflavin, 9.0 mg; calcium pantothenate, 11.0 mg; vitamin B_{12}, 13.0 Fg; niacin, 26.0 mg; choline chloride, 900 mg; vitamin K (Hetrazeen), 1.5 mg; folic acid, 1.5 mg; biotin, 0.25 mg; santoquin (Ethoxyquin), 125 mg; manganese, 55.0 mg; selenium, 0.1 mg; zinc, 50.0 mg; copper, 5.0 mg; iron, 30.0 mg.
†Same as above, but with no vitamin E or selenium.

Measurement of MDA and lipid hydroperoxides

For the analysis of MDA, samples of liver (2.5 g) were taken immediately after killing the bird and homogenized in a mixture of 12.5 mL of 5% (w/v) trichloroacetic acid in water and 1.25 mL of 0.5 g/L butylated hydroxytoluene (BHT) in methanol. The homogenate was then centrifuged at 2000 × *g* for 15 min at 4°C, and the supernatant was mixed with an equal volume of absolute ethanol and kept at −20°C for at least 2 h. The extract was then centrifuged at 2000 × *g* for 15 min at 4°C, and 2.0 mL aliquots of the clear supernatant were mixed with 1.0 mL of 0.2% thiobarbituric acid (TBA) in water and heated in a water bath at 85°C for 30 min in screw-capped tubes. The MDA–TBA complex was purified using C-18 reversed phase Sep-pak cartridges (Millipore Canada Ltd., Mississauga, Ontario) and measured by HPLC as previously described.[8,16]

For the analysis of lipid hydroperoxides, liver samples (2.5 g) were removed immediately after killing the bird and homogenized in 12.5 mL of BHT solution (0.5 g/L in methanol). The homogenates were centrifuged at 2000 × *g* for 30 min. at 4°C, and 5 mL aliquots of the clear supernatant were taken and dried with a stream of nitrogen. The levels of lipid hydroperoxides in the extracts were measured colorimetrically[17] at 353 nm using cumene hydroperoxide (Sigma Chemical Co., St Louis, MO) as the standard.

Measurement of antioxidant defense enzymes

Liver samples for the analysis of antioxidant defense enzyme activities were immediately removed after the bird was killed, frozen in liquid nitrogen, and stored at −80°C. GSHPx (EC 1.11.1.9) activity was measured by the method of Prohaska and Gutsch.[18] Samples of liver (0.5 g) were homogenized in 10 mL of cold 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 mM KCl and 0.15 mM EDTA. The homogenate was then centrifuged at 10,000 × g for 1 h at 4°C. Aliquots of 100 μL of the clear supernatant were used in the coupled assay procedure with glutathione reductase using t-butyl hydroperoxide. For the measurement of SOD (EC 1.15.1.1) activity, liver was homogenized in 10 volumes of cold 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA. The homogenate was centrifuged at 14,000 × g for 30 min at 4°C. The assay mixture contained 10 μM ferricytochrome C, 50 μM xanthine, 0.1 mM EDTA, 50 mM potassium phosphate (pH 7.8), 0.25 μM KCN, and sufficient xanthine oxidase to get an absorbance change of 0.025 OD units/min at 418 nm. Aliquots of liver extracts were added to the reaction mixture, and the percentage inhibition of reduction of cytochrome c was determined. One unit of SOD activity results in a 50% reduction in the rate of reduction of cytochrome c.[19] For the determination of catalase (EC 1.11.1.6) activity, 0.5 g of liver was homogenized in 5 mL of cold 0.01 M sodium phosphate buffer (pH 7.4), and the homogenate was centrifuged at 700 × g for 10 min at 4°C. Ethanol was added to the clear supernatant to a final concentration of 0.17 M and the mixture kept in ice water for 30 min. The sample was then diluted 100-fold with 0.01 M sodium phosphate buffer (pH 7.4), and Triton X-100 was added to a final concentration of 1.0%. The assay was carried out as reported by Cohen et al.[20] for mouse liver.

Statistical analysis

The data were analyzed using the general linear models procedure of SAS[21] using a model of strain, week, diet, and strain–diet interaction. Significant effects were separated using Duncan's multiple range test.[22]

Results

The average liver hemorrhage scores of birds for each week of the experiment are shown in *Figure 1A*. There was a significant effect of week, strain, and diet and a significant interaction between strain and diet on liver hemorrhage. There was a greater degree of liver hemorrhage in the UCD-003 birds than in normal birds by 2 weeks, and this increased in the UCD-003 birds when diet 2 was eaten. The hepatic levels of MDA in the different treatment groups of birds over the course of the experiment are compared in *Figure 1B*. Levels of MDA in the livers of all groups increased over the first 3 weeks of the trial and then decreased, also with significant effects of strain and diet. For diet 2, MDA levels in the livers of UCD-003 hens were higher than in the livers of normal birds ($P < 0.05$) at week 2, week 3, and week 4. The trend was the same for birds fed diet 1, but the MDA levels were not statistically different between strains of birds. Higher levels of MDA were found when the diet with no added selenium and vitamin E was fed than when the diet supplemented with selenium and vitamin E was fed at week 1 (in normal birds) ($P < 0.05$) and at week 2, week 3, and week 4 (in UCD-003 birds) ($P < 0.05$). The levels of lipid hydroperoxides in the livers of the two strains of birds fed the two different diets are

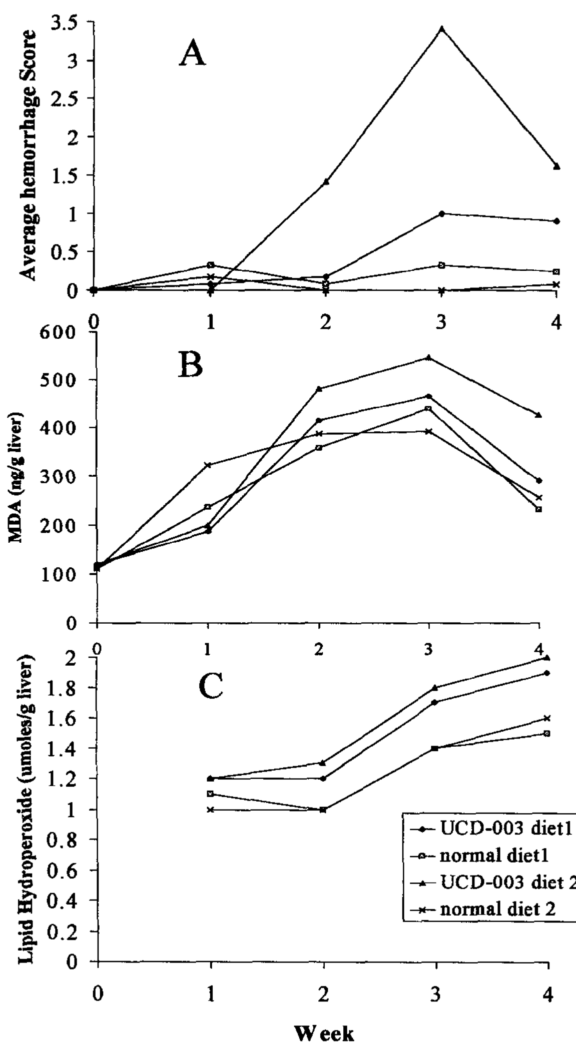

Figure 1 Effect of diet and strain on liver hemorrhage, MDA, and lipid hydroperoxide levels in the liver. (A) The average hemorrhage scores of livers from normal and UCD-003 birds over the course of the trial were assessed using a five-point scale. (B) Levels of MDA in the liver were determined weekly by HPLC analysis. (C) Levels of lipid hydroperoxides in the liver of normal and UCD-003 strain birds were determined as described in Materials and Methods. Diet 1 contains vitamin E at 11 IU/kg and selenium at 0.1 mg/kg and diet 2 has no added vitamin or selenium. Datapoints represent the average of measurements on six birds. MDA and lipid hydroperoxide levels were assayed in duplicate for each bird.

shown in *Figure 1C*. Lipid hydroperoxide levels increased in all birds over the course of the experiment and were higher the livers of UCD-003 birds than in normal birds.

The activities of the antioxidant defense enzymes GSHPx, SOD, and catalase in the liver of the two strains of birds fed the two different diets are presented in *Figure 2*. There was a significant effect of week and strain with no effect of diet on the activities GSHPx and SOD. Similar effects were seen if the enzyme activities were expressed on the basis of per gram of liver, per gram of protein, or total liver as a percentage of body weight. The activity of GSHPx decreased for the first week ($P < 0.05$) in all groups, as well as for the second week in UCD-003 birds ($P < 0.05$),

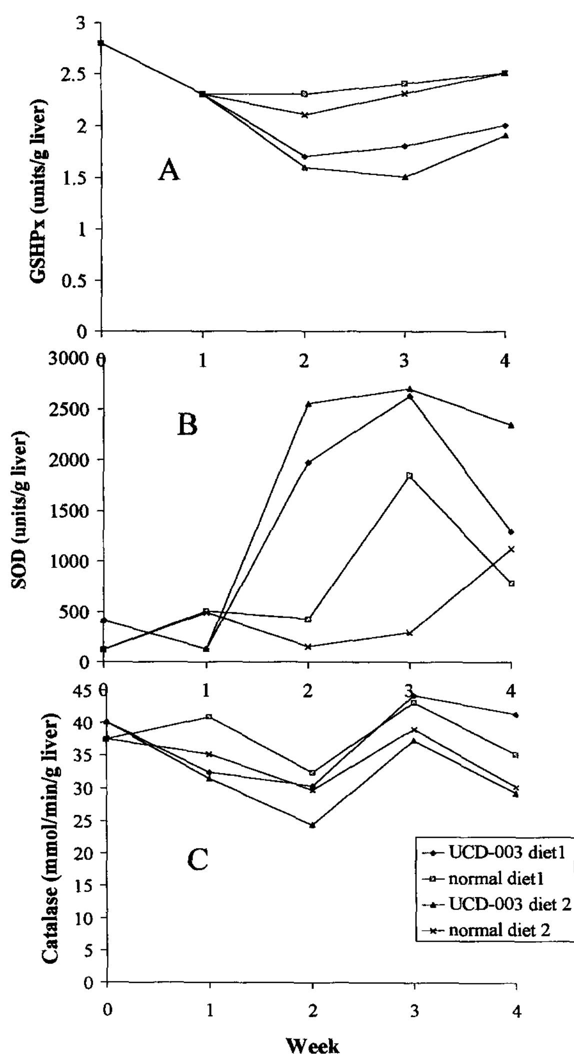

Figure 2 Effect of diet and strain on the antioxidant enzyme activities in the liver. The activity of glutathione peroxidase (GSHPx) (A), superoxidase dismutase (SOD) (B), and catalase (C) in the liver of normal and UCD-003 strain birds were determined weekly as described in Materials and Methods. Diet 1 contains vitamin E at 11 IU/kg and selenium at 0.1 mg/kg and diet 2 has no added vitamin or selenium. Datapoints for GSHPx and SOD represent the average of duplicate measurements on six individual birds, and datapoints for catalase represent measurements on two birds.

and then recovered (*Figure 2A*). The activity of SOD increased dramatically from week 2 in UCD-OO3 birds ($P < 0.05$; *Figure 2B*), while SOD activity in normal birds increased in week 3 in birds fed diet 1. There was a significant effect of week and diet, but no effect of strain on catalase activity (*Figure 2C*). Levels of catalase were generally lower in birds fed diet 2, but the changes in catalase activity were less dramatic than the changes seen in SOD and GSHPx.

Levels of MDA and lipid hydroperoxides in the feed did not change over the course of the experiment and were similar in the different diets ($P > 0.05$) (data not shown). There was no change in feed consumption in both strains of birds over course of the experiment ($P > 0.05$; data not shown). Egg production in the UCD-003 birds was lower than in the normal birds and averaged 68.8% and 82.1%, respectively, over the course of the trial. Egg production did not change over the course of the trial and was not affected by diet ($P < 0.05$).

Discussion

We have previously shown that the UCD-003 strain of birds is more susceptible to lipid peroxidation than normal birds following injection of iron.[8] The incidence of liver hemorrhage is also greater in UCD-003 birds than normal birds.[10] In the present work, higher amounts of lipid peroxidation and hemorrhage in the liver were found in UCD-003 birds than in normal birds in response to feeding diets containing high levels of PUFA. These effects were more pronounced when a diet without supplemental vitamin E and selenium was fed. It is thus apparent that increased lipid peroxidation occurs in conjunction with the development of liver hemorrhages in chickens.

The levels of MDA and lipid hydroperoxides and the activities of antioxidant defense enzymes are all potentially useful as indices of the extent of lipid peroxidation that has occurred in vivo. We have found that the levels of lipid hydroperoxides in the livers of both strains of birds increased over the course of the experiment, while the MDA levels increased for the first 3 weeks after feeding the PUFA diet and then decreased. This decrease in MDA levels after 3 weeks might be due to the elimination of MDA metabolites in the urine[23] or by the binding of MDA to macromolecules, such as proteins and nucleic acids, possibly resulting in cellular damage.[24,25] However, levels of MDA in the urine were not increased in vitamin E-deficient rats.[26] The usefulness of MDA alone as an index of *in vivo* lipid peroxidation has been questioned.[27]

We have found that the activities of SOD and GSHPx changed in response to feeding diets containing high levels of PUFA, but this change was less dramatic in the livers of normal birds than in the livers of UCD-003 birds. The increase in SOD activity was not unexpected due to the involvement of this enzyme in the conversion of superoxide to hydrogen peroxide. The dramatic increase in activity seen in the UCD-003 birds compared to the normal birds may be due to increased production of superoxide in the UCD-003 strain. The decrease in GSHPx activity may be due in part to insufficient selenium, but may also be the result of reaction with certain products of lipid peroxidation that may inhibit this enzyme in cells, particularly in the UCD-003 birds. Lipid hydroperoxides and aldehydes such as MDA may be concentrated in specific areas and block the essential sulfhydryl groups on enzymes.[28,29] The activities of aldehyde dehydrogenase, glucose-6-phosphatase, and cytochrome P450 in microsomes were inhibited by increased lipid peroxidation from iron and ascorbate, and this inactivation was prevented by antioxidants.[30] Tissue levels of MDA and lipid hydroperoxides and the activities of the antioxidant defense enzymes may change at different rates over time in response to an oxidative stress. To use these parameters as an index of in vivo lipid peroxidation, measurements of more than one timepoint are necessary. This is illustrated by our results, which indicate the differences in the accumulation of MDA and lipid hydroperox-

ides and in the changes in activity of GSHPx and SOD over the course of the experiment.

FLHS is a metabolic disorder of laying hens that is characterized by the accumulation of a large amount of fat in the liver that predisposes the birds to the development of liver hemorrhages. The fat can occupy the entire parenchyma cytoplasm and may contribute to structural weakness that renders the liver more liable to hemorrhage.[31] However, large amounts of lipid can also be found in the livers of birds showing no hemorrhage. We have previously proposed that increased peroxidation of the lipid in the liver leads to the rupture of cell membranes and hemorrhage.[7] The present study, in which feeding a diet containing high levels of PUFA and not supplemented with vitamin E and selenium resulted in higher amounts of liver hemorrhage than the same diet supplemented with vitamin E and selenium, supports this hypothesis. We conclude that appropriate levels of antioxidants are necessary to protect laying hens against the development of liver hemorrhages.

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