Influence of Various Flavonoids and Simple Phenolics on Development of Exudative Diathesis in the Chick†

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Three-day-old broiler chicks were fed a diet containing low concentrations of selenium and vitamin E for 21 days to produce exudative diathesis. Dietary supplements (1000 mg/kg) of various flavonoids and simple phenolics were tested for their protectiveness against the disorder. Two of the flavonoids, rutin and silymarin, markedly reduced the incidence and severity of exudative diathesis, while three other flavonoids (catechin, quercetin, and morin) and both of the simple phenolics tested (*p*-coumaric and ferulic acids) provided no protection. The results indicated that protection against exudates by rutin and silymarin was not the result of their direct action as antioxidants or enhanced utilization of selenium or vitamin E. It is generally considered that flavonoids are successful in reducing vascular disorders in humans by strengthening fibrous membranes. A similar effect may have occurred here in reducing exudative diathesis, a disorder that results from a pathological increase in capillary permeability.

INTRODUCTION

In addition to "primary" metabolites associated with growth and development, plants produce a wide variety of "secondary compounds" that appear to have no primary metabolic function but may have a role in plant defense against disease and predation (Blum, 1986). The flavonoids are a widely distributed group of plant secondary compounds that have attracted interest, as potential natural antioxidants both for inhibiting oxidative deterioration of foodstuffs and for providing beneficial metabolic effects in animals (Herrmann, 1976; Kühnau, 1976; Singleton, 1981; Fahey and Jung, 1989). For example, the antioxidant properties of flavonoids in vegetable foodstuffs have an important role in prolonging shelf life and keeping quality of vegetable products (Herrmann, 1976; Kühnau, 1976). At the same time their antioxidant activities help maintain the taste and quality of accompanying animal products by inhibiting oxidative deterioration of the animal lipids. Regarding the metabolism of flavonoids in animals, numerous beneficial effects have been reported, some of which appear to be related to their antioxidant and chelating properties (Fahey and Jung, 1989). These have included protective effects against hepatoxic agents (Fraga et al., 1987), lipid peroxidation in human platelets (Koch and Löffler, 1985), inhibition of lipoxygenase and prostaglandin synthetase activities (Larson, 1988), and reduction of capillary fragility and permeability problems in humans (Fahey and Jung, 1989). There has been extensive research on this latter effect. Indeed, flavonoid drugs have been widely used in medical practice for over 40 years for circulatory disorders involving capillary dysfunction (Jaeger et al., 1988). However, there appear to have been no studies on the effects of flavonoids on the development of vitamin E–selenium disorders in animals. This is surprising in that flavonoids represent a potent dietary source of antioxidant activity (Kühnau, 1976) and many of the vitamin E–selenium deficiency conditions [e.g., exudative diathesis (ED), encephalomalacia, and nutritional muscular dystrophy] are influenced by various synthetic antioxidants (Combs and Scott, 1974; Draper, 1980) such as *N*,*N*^{1}-diphenyl-*p*-phenylenediamine (DPPD), 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline (ethoxyquin, santoquin) (Monsanto Chemical Co., St. Louis, MO), and 3,7-bis(dimethylamino)phenazathionium chloride (methylene blue).

In view of the above, we fed newborn chicks an ED-producing diet and tested the efficacy of various flavonoids and phenolic compounds in preventing the disorder. Exudative diathesis was chosen as this vitamin E–selenium condition develops as a consequence of a pathological increase in capillary permeability (Dam et al., 1957) and in view of the reported effectiveness of flavonoids in preventing or alleviating capillary fragility and permeability conditions (Fahey and Jung, 1989).

MATERIALS AND METHODS

Materials. The silymarin group was purchased from Aldrich Chemical Co., Milwaukee, WI (according to the supplier, the silymarin group is comprised of mainly three isomers—silybin, which is the major component, silydianin, and silychristin; the relative amounts were not given). (+)-Catechin, *p*-coumaric acid, ferulic acid, morin, quercetin dihydrate, rutin trihydrate, and D-α-tocopheryl acetate were purchased from Sigma Chemical Co., St. Louis, MO. The chemical structures are shown in Figure 1.

Experimental Procedures. Ninety 3-day-old unsexed chicks of the Centre for Food and Animal Research (CFAR) meat strain 31 (Chambers et al., 1984) were individually banded, fed a commercial starter diet for 3 days, and then randomized into 18 groups of 5 chicks each, providing 9 treatment groups in duplicate. Each group of five chicks was then randomly assigned a pen in the battery brooders. The subsequent feeding trial was for 3 weeks. Housing consisted of wire-floored battery brooders, electrically heated, with 23 h of light provided daily. Feed and water were provided for ad libitum consumption.

The basal diet (Table I) used to produce ED essentially was that of Hassan (1990), except here isolated soy protein was substituted for extracted soybean meal and vitamin E-stripped corn oil replaced cottonseed oil, in which vitamin E had been removed by a distillation process. The vitamin E and selenium concentrations were very low (0.5 and 0.02 mg/kg, respectively) and similar to levels reported by Hassan (1990). Adequate amounts of methionine (2.4 g/kg) and ethoxyquin (0.08 g/kg) were included in the basal diet to protect the chicks against nutritional muscular dystrophy and nutritional encephalomalacia.

The dietary treatments were controls (ED), (+)-catechin, *p*-coumaric acid, ferulic acid, morin, quercetin dihydrate, rutin trihydrate, silymarin, and a natural source of vitamin E as D-α-

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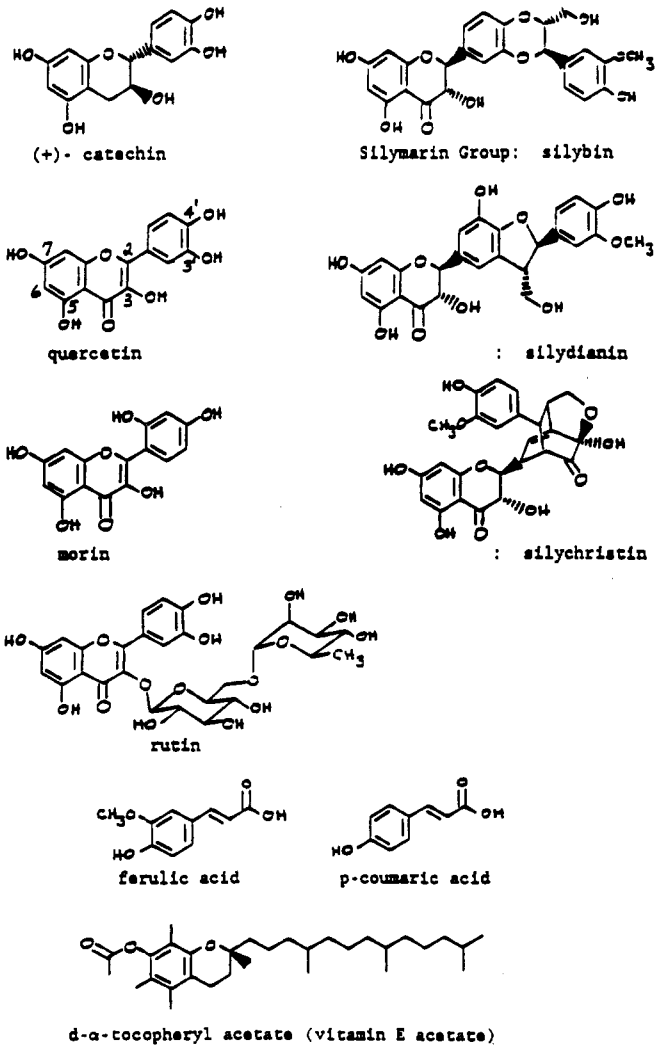

Figure 1. Flavonoids and simple phenolics tested for protection against exudative diathesis.

Table I. Composition of Basal Diet for Producing Exudative Diathesis

ingredient	
glucose, g/kg	569.02
torula yeast,[b] g/kg	140.00
casein, vitamin-free,[b] g/kg	90.00
isolated soy protein,[b] g/kg	80.00
cellulose,[a] g/kg	30.00
corn oil, vitamin E-stripped,[b] g/kg	30.00
macro salts premix,[c] g/kg	40.00
micro salts premix,[c] g/kg	2.00
vitamin premix,[c] g/kg	10.00
L-arginine hydrochloride,[a] g/kg	3.00
DL-methionine,[a] g/kg	2.40
glycine,[a] g/kg	2.00
L-lysine hydrochloride,[a] g/kg	1.50
ethoxyquin,[a] g/kg	0.08
vitamin E content, mg/kg	0.50
selenium content, mg/kg	0.02

[a] Purchased from Sigma Chemical Co. [b] Purchased from ICN Biochemicals, St. Laurent, Quebec. [c] Macro salts premix provided (g/100 g of diet) the following: $CaHPO_4 \cdot 2H_2O$ 1.87, $CaCO_3$ 0.65, KH_2PO_4 0.69, MgO 0.08, NaCl 0.60. Micro salts premix (mg/100 g of diet): $FeSO_4 \cdot 7H_2O$ 41.4, $MnSO_4 \cdot H_2O$ 33.3, KI 0.26, $CuSO_4 \cdot 5H_2O$ 1.67, ZnO 6.0. Vitamin premix (mg/100 g of diet): niacin 5, calcium pantothenate 2, thiamin hydrochloride 1, riboflavin 1, pyridoxine hydrochloride 0.45, folic acid 0.4, menadione 0.05, D-biotin 0.02, vitamin B_{12} 0.002, vitamin A palmitate 500 IU, vitamin D_3 38 ICU, choline chloride 150.

tocopheryl acetate. The flavonoids and phenolics were included in the basal diet at 1000 mg/kg, and vitamin E was included at 50 mg/kg. The 1000 mg/kg treatment figure was chosen as it is the concentration at which the antioxidant diphenyl-*p*-phenylenediamine prevents encephalomalacia (Singsen et al., 1955) and ethoxyquin prevents muscular dystrophy (Machlin et al., 1959) in chicks.

The development of ED in chicks was determined by daily visual examination for external symptoms, as described by Dam and Glavind (1938). These symptoms included edema of the breast, abdominal regions, wings, jowls, neck, and feet, characterized by a bluish discoloration under the skin caused by leakage of hemoglobin from the capillaries and the formation of hemoglobin breakdown products. Chicks that showed clinical signs of ED were killed by carbon dioxide asphyxiation and autopsied immediately, as were chicks killed at the end of the trial. The severity of ED in each chick was a subjective estimate by visually inspecting seven sites at autopsy (i.e., breast muscle, wings, neck, thigh, crop, abdominal region, feet) and scoring each site according to the following values: severe exudates = 10; appreciable = 6; slight = 3; none = 0. The scores were combined for overall ED severity, with 70 the highest possible score. Exudative diathesis was most severe in the breast muscle, neck, and abdominal region. The disorder usually was slight or did not occur in the wings, thigh, crop, and feet. Thus, most of the severity score was derived from the breast muscle, neck, and abdominal areas. Chicks with ED that die frequently show typical signs only in these areas.

Blood samples were taken in heparinized tubes prior to killing the chicks and livers removed at post-mortem. Plasma, separated by centrifugation, and livers were stored at −20 °C.

Analyses. Vitamin E in 1 mL of plasma was isolated with the lipid material by extraction with a mixture of 2 mL of hexane, 4 mL of ethanol, and 1 mL of deionized water. The hexane phase was evaporated to dryness under nitrogen and the residue dissolved in 2 mL of hexane. An aliquot was analyzed by HPLC using the method described by Hidiroglou (1989). For hepatic vitamin E, 1 g of liver was homogenized with a mixture of 2 mL of water and 10 mL of ethanol. After centrifugation, the supernatant was extracted with 10 mL of hexane; a 5-mL aliquot of the extract was evaporated to dryness under nitrogen, dissolved in 2 mL of hexane, and analyzed for vitamin E by HPLC, as for plasma.

Selenium concentrations in liver and plasma were determined according to the method of Hoffman et al. (1968).

Statistics. A randomized, two-way design was used with nine treatments of five chicks each, run in duplicate. Data were subjected to ANOVA and treatment means separated by Duncan's multiple-range test (Steel and Torrie, 1980) using 5% probability.

RESULTS AND DISCUSSION

Weight gains were not affected appreciably by the various dietary treatments (Table II) or by gender (not shown). The growth data were based, however, on a relatively small number of animals in some lots, particularly for the third week, as the result of chicks being killed when they developed signs of exudative diathesis (ED). At 2 weeks, only the basal diet and silymarin lots had significantly ($P < 0.05$) lower weight gains than for other treatments. After 3 weeks, the ferulic acid and morin lots had the lowest gains ($P < 0.05$). The weight gain data indicated that none of the flavonoids or phenolics was highly toxic at the 1000 mg/kg intake level. Feed consumption data were obtained for each pen of five chicks with two pens per treatment. The data were similar for duplicate pens and feed efficiencies similar for all treatments (not included). There was no relationship found between body weight of a chick and development of ED.

None of the chicks on the vitamin E-supplemented diet (50 mg/kg) developed ED (Table II). Birds fed the control diet developed the disorder rapidly. Two of the ten chicks showed signs of ED within 2 weeks and were killed, and an additional four birds were observed to have developed the disorder during the third week. At the end of the experiment (21 days), ED was found at autopsy in three of the four remaining chicks. Hassan (1990) reported a

Table II. Influence of Various Flavonoids and Simple Phenolics on Development of Exudative Diathesis and on Concentrations of Selenium and Vitamin E in Plasma and Liver[a]

dietary treatment	no. of chicks[b] male	no. of chicks[b] female	chicks with ED[c] at 2 weeks	chicks with ED[c] at 3 weeks	3-week incidence as decimal[g]	av severity[d] of ED at 3 weeks	av wt gain, g, of survivors at 2 weeks	av wt gain, g, of survivors at 3 weeks	av selenium concn[e] in plasma, μg/mL	av selenium concn[e] in liver, μg/g of DM	av vitamin E concn[e] in plasma, μg/mL	av vitamin E concn[e] in liver, μg/g of DM
nil (basal diet)	6	4	2	9	0.9A	27.0A	290B (8)	540AB (4)	0.011	0.32	0.16AB	1.35A
catechin	7	3	2	8	0.8A	30.9A	317AB (8)	492B (3)	0.009	0.27	0.11B	0.98B
p-coumaric acid	4	6	2	8	0.8A	25.8A	323AB (8)	548AB (4)	0.010	0.29	0.14AB	0.58C
ferulic acid	4	6	2	9	0.9A	36.1A	302AB (8)	487B (3)	0.008	0.25	0.10B	0.59C
morin	6	4	2	10	1.0A	32.7A	310AB (8)	478B (3)	0.009	0.27	0.15AB	1.16AB
quercetin dihydrate	7	3	2	9	0.9A	31.4A	319AB (8)	602A (3)	0.010	0.26	0.15AB	0.65C
rutin trihydrate	5	5	1	4	0.4B	8.1B	305AB (9)	536AB (8)	0.012	0.26	0.16AB	1.02B
silymarin	4	6	1	4	0.4B	8.9B	286B (9)	512AB (8)	0.008	0.33	0.18A	1.11AB
vitamin E	5	5	0	0			338A (10)	586AB (10)	0.007	0.27	15.9	77.4
SEM[f]					0.12	5.3	15.4	36.1	0.002	0.03	0.024	0.11

[a] Means are for 10 chicks for each treatment (2 groups of 5 chicks, each group randomly assigned to a battery pen), except for average weight gains, which were for chicks remaining on experiment, as shown in parentheses. Chicks were killed when they showed signs of exudative diathesis. Vitamin E concentrations in plasma and liver of birds fed vitamin E were not included in statistical analyses. [b] At start of experiment. Gender determined at autopsy. [c] ED, exudative diathesis. Data at 2 weeks are for chicks that showed signs of ED and were killed and at 3 weeks for all chicks. Incidence as mean is group average at 3 weeks for those with (1.0) or without (0.0) ED. [d] Average severity of ED for all chicks in lot using scoring system described under Materials and Methods. [e] Values for liver dry matter. [f] SEM, standard error of mean. [g] ABC, means in the same column with different letters are significantly different at $P < 0.05$.

similar rate of development for ED in chicks fed this diet. He also found that birds developed the disorder 4–11 days earlier if hatched from eggs derived from selenium-deficient hens. Our other treatments had no appreciable effect on the incidence of ED at 2 weeks (Table II). However, at 3 weeks only four of ten chicks had ED when given either the rutin or silymarin treatments, whereas the control, phenols, and other flavonoids had an incidence between 80% and 100%. Severity scores for ED were similar for rutin and silymarin treatments, and scores for both were significantly ($P < 0.05$) lower than for all other lots (Table II). For all treatments, ED was found at autopsy to the greatest extent in the abdominal regions and breast muscle and then in decreasing order in the neck, thigh, wings, feet, and crop. All of the sites showed a similar reduction in ED severity by rutin and silymarin; i.e., no particualr site was protected to a greater extent.

Selenium concentrations in plasma and liver were very low for all groups, and except for a tendency for lower plasma selenium for the ferulic acid, silymarin, and vitamin E birds, they were unaffected by the various treatments. The values were similar to those reported by Hassan et al. (1987) for selenium-deficient chicks fed adequate vitamin E. These results indicate that the partial protection against ED provided by rutin and silymarin was not caused by an improvement in selenium utilization. However, plasma glutathione peroxidase (GSH-P_x, glutathione-hydrogen peroxide oxidoreductase, EC 1.11.1.9) activity, which was not measured in this study, might have been a more reliable criterion for evaluation of selenium bioavailability than plasma and liver selenium concentrations. It has been shown that supplementing low-selenium diets with selenium increases plasma GSH-P_x while preventing ED (Combs and Scott, 1974). Thus, in this study it is possible that rutin and silymarin may have reduced onset of ED by enhancing the synthesis of this enzyme (subsequent studies will investigate this possibility). In this regard, Combs and Scott (1974) found that a high intake of ethoxyquin prevented ED while simultaneously increasing GSH-P_x activity and suggested that this may have been the result of increased selenide production, a form readily incorporated into GSH-P_x. A popular concept for major metabolic roles for selenium and vitamin E involves selenium in GSH-P_x destroying peroxides in the cytosol of cells and vitamin E preventing peroxidation of unsaturated lipids in cell membranes (Noguchi et al., 1973).

The plasma vitamin E concentrations were low and similar for all dietary treatments that did not include supplemental vitamin E (Table II). There was a tendency to lower values for the ferulic acid and catechin treatments and slightly higher concentrations for silymarin, but, in general, plasma vitamin E concentrations did not correspond to protection against ED. Control chicks had very low levels of vitamin E in the liver, which corresponded closely to those reported by Hassan (1990) for chicks with ED. Notably, none of the flavonoid or phenol treatments resulted in higher liver vitamin E concentrations than the controls, indicating that any antioxidant properties of these compounds did not protect vitamin E during absorption, systemic transport, or storage. Vitamin E acetate, the form fed, is considered to be stable during gastrointestinal transit. Chicks fed ferulic acid or quercetin had very low liver vitamin E levels and high severity of ED, but vitamin E concentrations for rutin or silymarin treatments were not elevated in accordance with their inhibition of ED. Overall, there was no apparent relationship between plasma and liver vitamin E concentrations and protection against ED.

Rutin and silymarin may have had a direct effect in reducing ED, rather than by improving the efficacy of inadequate amounts of selenium or vitamin E. Very little is known of silymarin metabolism in animals. This flavonoid complex is derived from the thistle *Silybum marianum*, and very little occurs in feeds or foods. It has been proven to be effective in humans against the hepatotoxicity of many xenobiotics (Bindoli et al., 1977; Fraga et al., 1987), and in animals it has been shown to have cell-regenerating activity and was an effective inhibitor of lipid peroxidation (Campos et al., 1988). It was included here in view of its pharmacological uses and antiperoxidative action (Bindoli et al., 1977). On the other hand, there is considerable information on the metabolism of the flavonoids we tested (i.e., rutin, quercetin, morin, and catechin) which occur widely in foods and feeds. The average daily intake of flavonoids by humans in the United States has been estimated at 1 g (Pierpoint, 1986). Rutin would represent a relatively small fraction of total food flavonoid intake (Harborne, 1979), but health store recommendations of 1 g/day could increase this consid-

erably (MacGregor, 1984). Natural grower rations for poultry contain 200–900 mg/kg polyphenolics of which rutin and other flavonoids glycosides are commonly present (Fahey and Jung, 1989; Harborne, 1979; Kühnau, 1976). When ingested, flavonoids have two metabolic fates in animals. A portion of the aglycons and glycosides is absorbed into the animal essentially unchanged, whereas another part is metabolized extensively by the intestinal microflora (Kühnau, 1976). In the latter case, bacterial catabolism in the gastrointestinal tract results in removal of sugar residues and degradation of aglycons to phenolic acids or their lactones which are absorbed and excreted in the urine or recycled to the gut via the bile. Since phenolic acids formed by bacterial catabolism of flavonoids are normal body constituents or breakdown products of aromatic amino acids and amines, it is considered highly unlikely that they are responsible for the physiological effects of flavonoids (Kühnau, 1976). Animal tissues also catabolize part of the absorbed aglycons to breakdown products similar to those produced by microorganisms but much less extensively (Singleton, 1981; Fahey and Jung, 1989). As glycosidases are absent in animal tissues, it thus remains that either the intact glycosides or aglycons that were absorbed were responsible for the biological effects. In the present study, unaltered rutin was likely inhibiting ED since rutin is a glycoside of quercetin and had ED protectivity, whereas the aglycon quercetin was ineffective.

From what is presently known about flavonoid metabolism in animals, there are several effects that appear most likely to account for rutin and silymarin providing partial protection against ED. One possibility is that the flavonoids acted directly as antioxidants. Bindoli et al. (1977) showed that silymarin strongly inhibited peroxide formation in rat liver mitochondria and microsomes, an activity 10-fold higher than that of α-tocopherol. They attributed the effect to free-radical scavenger activity. In our work, if rutin were acting as an antioxidant, it would appear that the structural requirements for antioxidant activity were different from those established using in vitro studies. For example, in lipolytic systems, the main antioxidant activity has been considered to reside in the 4′ and 3′,4′ hydroxyl groups (see Figure 1) and in the 2,3 double bond (Kühnau, 1976). A free 3-OH was not indispensable, and in general the antioxidant effect increased with the number of hydroxyls (Kühnau, 1976). However, in this study, quercetin met most of these structural requirements for in vitro antioxidant activity but was ineffective against ED. Rutin was protective but had the same structure as quercetin except for the 3-rhamnoglucose group. Silymarin was structurally similar to quercetin but lacked the 2,3 double bond and 3′,4′ hydroxyl groups but had an additional coniferyl alcohol residue, which may have contributed to protectiveness against ED.

Alternatively, rutin and silymarin may have provided partial protection against ED via the formation of flavonoid metal chelates, either by inactivating copper-requiring enzymes, such as ascorbic acid oxidase, polyphenol oxidases, and hyaluronidase, or by promoting cross-links in elastin and collagen (Kühnau, 1976). This latter effect, which results in the strengthening of fibrous membranes, has frequently been ascribed to flavonoids as a rationale of their therapeutic success in treating vascular disorders (Kühnau, 1976).

In conclusion, rutin and silymarin markedly reduced the onset of ED in chicks to 24 days of age, whereas other flavonoids (catechin, quercetin, and morin) and the simple phenolics (*p*-coumaric and ferulic acids) tested had no protective activity. Further research will be required to determine the mode of action of rutin and silymarin in inhibiting the development of this selenium–vitamin E deficiency disorder.

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