Effect of Santoquin® and Oxidized Fat on Liver and Intestinal Glutathione in Broilers

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ABSTRACT Experiments were conducted to determine effects of Santoquin® (ethoxyquin) and oxidized fat on liver and intestinal reduced (GSH) and oxidized (GSSG) glutathione, and pulmonary hypertension syndrome (PHS) mortality. Male broilers were randomly assigned in a 2 × 2 factorial consisting of 3.5% normal (NF) or oxidized (OxF) fat with or without ethoxyquin (E). Body weights and feed intake were monitored weekly, and tissues obtained at 3 and 7 wk for GSH and GSSG analysis. Compared to the NF group, NF/E gained more weight during the starter (0 to 3 wk), but not the grower (4 to 7 wk) period. Birds fed NF/E or NF exhibited greater feed efficiency in the starter period and greater gains during the starter and grower periods than birds fed OxF or OxF/E. No differences in PHS mortality between treatments were observed. Birds fed OxF exhibited lower liver GSSG at 3 wk than the other groups, but there were no differences in liver GSH. Duodenal GSH was higher in birds fed OxF/E than in birds of NF group at 3 and 7 wk. Ileal GSH was higher at 3 wk in OxF/E birds than in OxF birds, but no differences were observed at 7 wk. All tissues exhibited higher GSH levels at 7 wk than at 3 wk. Birds fed ethoxyquin, regardless of fat source, exhibited higher duodenal GSH at 3 and 7 wk and higher ileal GSH at 3 wk than birds that did not receive ethoxyquin. Higher GSH would be beneficial by enhancing protection of intestinal cells to deleterious effects of toxins or other forms of oxidative stress.

(*Key words*: ethoxyquin, broiler, liver, intestines, glutathione)

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INTRODUCTION

Santoquin®[1] (ethoxyquin) is a widely used feed grade antioxidant that can prevent the oxidation of lipids and lipid-soluble components in the feed (Waldroup *et al*., 1961; Bartov and Bornstein, 1981; Harms *et al*., 1984; Cabel and Waldroup, 1989). Feeding oxidized or rancid fats has been shown to have deleterious effects, including increased incidence of encephalomalacia (L'estrange *et al*., 1966), increased peroxidation of cell membranes (Asghar *et al*., 1989; Hayam *et al*., 1993), and decreased growth or feed efficiency (Nakamura *et al*., 1972; Reddy and Tappel, 1974; Inoue *et al*., 1984; Cabel *et al*., 1988). Conversely, ethoxyquin prevented or lowered muscular dystrophy in rats (Gabriel *et al*., 1980), encephalomalacia induced by vitamin E deficiency (Machlin and Gordon, 1960), exudative diathesis (Combs and Scott, 1974), and sterility in female rats (Draper *et al*., 1964). Preventing oxidation of dietary fats is important for optimal growth and feed efficiency in broilers. Increased intake of peroxides lowered body weights and feed efficiency, whereas broilers fed diets containing 125 ppm ethoxyquin and 7 meq peroxide/kg feed exhibited similar feed efficiencies and weight gains as broilers fed 125 ppm ethoxyquin alone (Cabel *et al*., 1988).

Lipid peroxidation (Asghar *et al*., 1989) and cell membrane rigidity (Hayam *et al*., 1993) due to feeding of oxidized fats are indicative of damage to cell lipids. Protection against cellular oxidation mediated by chemical radicals, toxins, and other mediators of oxidative stress is afforded by a host of endogenous antioxidants (e.g., Frei, 1994). Glutathione (GSH), an antioxidant found in millimolar concentrations in many tissues, plays critical roles in detoxification, protection of cells from oxidative stress, and in maintenance of cellular redox balance (Meister, 1984). Glutathione provides protection against cellular oxidants through nonenzymatic interactions and enzymatic-mediated mechanisms. Glutathione peroxidase reduces peroxides to water or lipid alcohols by catalyzing the release of hydrogen from the cysteinyl moiety of GSH. Thus, GSH might be particularly important in protecting cells from oxidative stress mediated by high levels of peroxides in the feed. Indeed, improved growth performance observed with ethoxyquin (Cabel *et al*., 1988) could be partially

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[1]Santoquin® is a registered trademark of Monsanto in the U.S. and other countries.

TABLE 1. Dietary proximate analysis for starter and grower diets[1]

Item	Starter	Grower
	(%)	
Crude protein	21.2	18.8
Moisture	12.2	11.2
Fat	6.3	6.3
Fiber	2.0	1.9
Ash	5.6	5.1
Amino acids		
Met	0.32	0.31
Cys	0.39	0.36
Lys	1.13	0.95
Arg	1.34	1.17

[1]Proximate analysis provided by Novus, International Inc., St. Louis, MO 63304. Analysis was made on the normal fat diet only. All other components, with the exception of fat, were the same for both the oxidized and normal fat diets.

mediated by GSH, as elevations in tissue sulfhydryl levels (which include GSH) have been observed in mice (Kim, 1984) and rats (Gavino *et al.*, 1985) receiving ethoxyquin. To our knowledge, the effect of ethoxyquin on tissue GSH levels has not been demonstrated in the broiler chicken, however. Therefore, the major objective of this study was to determine the effects of ethoxyquin and oxidized fat on liver and intestinal GSH levels. The intestines were chosen due to their intimate contact with digesta, whereas the liver was chosen due to its central role in GSH metabolism (Meister, 1984). A secondary objective was to determine the effect of ethoxyquin and oxidized fat on the incidence of pulmonary hypertension syndrome (PHS, ascites), as oxidative stress may play a role in the pathophysiology of this metabolic disease (Enkvetchakul *et al.*, 1993; Bottje *et al.*, 1995; Bottje and Wideman, 1995).

MATERIALS AND METHODS

Animals

Commercial broiler males (Cobb 500) were obtained from a local hatchery.[2] A total of 400 birds were randomly assigned to diet treatments and placed in floor pens within environmental chambers (25 chicks per pen, four pens per chamber). The birds were randomly assigned in a 2×2 factorial consisting of normal fat (NF) and oxidized fat (OxF) included at 3.5% of the diet, and 0 or 125 ppm Santoquin® (ethoxyquin, E). The birds were maintained on wood shavings litter and provided *ad libitum* access to water and feed. At 1 wk, the number of birds per pen was decreased to 20 by culling those with low body weights or deformed legs. Starter diets were provided for the first 3 wk and grower diets from 4 to 7 wk. Proximate analysis of the starter and grower diets is provided in Table 1. Feed intake and bird weights were determined weekly.

Protocol

Cool temperatures were used to induce pulmonary hypertension syndrome (PHS) as described by Wideman *et al.* (1995). The chicks were brooded at 32 and 28 C for Weeks 1 and 2. During Week 3, chamber temperatures were lowered from 28 to 15 C and maintained between 10 and 15 C during the remainder of the experiment. Birds that died during the experiment were necropsied to examine for symptoms of PHS, including the presence of ascites fluid and generalized venous congestion.

At 3 and 7 wk of age, 12 broilers per diet (3 per pen) were randomly selected for blood and tissue analysis. Five milliliters of blood was obtained by cardiac puncture directly into a heparinized syringe and centrifuged at 10,000 rpm for 2 min. The plasma was immediately frozen in liquid nitrogen and stored at –80 C until analysis. A microhematocrit was obtained from blood obtained from the wing vein. Birds were killed by cervical dislocation and portions of the liver, upper duodenum, and mid-ileum were obtained. Intestinal segments (~10 cm) were cut longitudinally and rinsed in saline to remove intestinal contents and blotted to remove surface moisture. All tissues were then immediately frozen in liquid nitrogen. Frozen tissues were stored at –80 C until analysis. The right ventricle (RV) and left ventricle plus septum (LV+S) were weighed to obtain the right ventricular weight ratio, which is correlated with pulmonary pressure (Burton *et al.*, 1968).

Chemical Analysis

Glutathione. Tissue and blood levels of reduced (GSH) and oxidized (GSSG) glutathione were determined by HPLC (Fariss and Reed, 1987). Briefly, perchloric acid precipitation of proteins was followed by reaction of iodoacetic acid with thiols to form S-carboxy-methyl derivatives and derivatization of amino groups in the supernatant with 1-fluoro-2,4-dinitrobenzene. Derivatized thiols were separated by ion-exchange column chromatography as previously described (Bottje *et al.*, 1991). Glutathione and GSSG were identified by retention times of authentic standards, and concentrations calculated from peak integrated areas.

Plasma Lipid Peroxides. Plasma lipid peroxides were quantified colorimetrically using a commercially available assay kit.[3] In this assay, hemoglobin catalyzes the reaction of hydroperoxides with a methylene blue derivative forming equal molar concentrations of methylene blue. A high correlation between this assay and the iodometric method has been reported (Pervaiz *et al.*, 1992).

Statistical Analysis

A two-way ANOVA was performed to determine differences between treatment groups, as well as main

[2]Randall Road Hatchery, Tyson Foods, Inc. Springdale, AR 72762.
[3]Kit No. CC-004, Kamiya Biomedical Co., Thousand Oaks, CA 91360.

TABLE 2. Initial dietary peroxides levels for starter and grower diets, and weight gain, feed intake, and feed efficiency for the starter (0 to 3 wk) and grower (4 to 7 wk) periods in broilers fed diets containing 3.5% normal fat (NF) or oxidized (OxF) fat with or without 125 ppm ethoxyquin (E)

Variable	Period	Diet				Pooled SE
		NF/E	NF	OxF/E	OxF	
Initial dietary peroxide concentrations,[1] meg/kg						
	Starter	0.8	0.4	11.6	17.6	. . .
	Grower	0.6	0.8	32.4	39.4	. . .
Weight gain,[2] kg						
	Starter	0.638[a]	0.600[b]	0.540[b]	0.551[b]	0.012
	Grower	2.141[a]	2.247[a]	2.019[b]	1.908[b]	0.072
Feed intake,[2] kg/bird/period						
	Starter	1.031[a]	0.997[ab]	0.952[b]	0.965[b]	0.017
	Grower	4.272[a]	4.397[a]	3.995[b]	3.973[b]	0.081
Feed efficiency,[2] g gain:g feed						
	Starter	0.619[a]	0.602[a]	0.567[b]	0.572[b]	0.008
	Grower	0.501	0.511	0.506	0.408	0.014

[a–c]Means within a row with no common superscript differ significantly ($P < 0.05$).

[1]Analysis provided by Novus International, Inc. St. Louis, MO 63304.

[2]Means ± SE (four pens of 15 to 20 birds per pen) adjusted for dead bird mortality weights.

effects of age and diet by age interactions using the General Linear Models procedure of SAS® (SAS Institute, 1985). Data were subjected to analysis of variance for unequal means and are expressed as the mean ± standard error. Chi-square analysis was used to assess differences in PHS mortality between treatments. A probability level of less than or equal to 0.05 was considered significantly different.

RESULTS

Analysis of diets for peroxides revealed values ≤ 0.8 meq/kg for NF or NF/E diets compared to ≥ 11 meq/kg for OxF/E or OxF diets (Table 2). Peroxide values in OxF with or without E were at least twice as high in grower diets as in the starter diets and the OxF/E diets had peroxide values that were 6 to 7 meq/kg less than those of diets containing OxF alone.

Growth performance for the starter and grower periods are also shown in Table 2. During the starter period, birds fed NF/E gained more weight than birds in the other treatment groups. Feed intake during the starter period was higher in birds fed NF/E than in birds of either OxF/E or OxF groups, but was not different than that of birds fed NF alone. Weight gain and feed intake were higher during the grower period in the NF/E and NF groups than in the OxF/E or OxF groups. Feed efficiency was higher during the starter period for birds fed NF/E or NF than for those fed OxF/E or OxF, but there were no differences in feed efficiency between groups during the grower period.

Hematocrit, the right ventricular weight ratio (RV: LV+S), and plasma lipid peroxides obtained in birds randomly sampled at 3 and 7 wk are shown in Table 3. The NF birds exhibited higher hematocrit values than NF/E birds at 3 wk. There were no differences in RV: LV+S at 3 wk of age, but at 7 wk, RV/LV+S was higher in NF/E birds than in OxF birds. No differences in lipid peroxide values between dietary treatment groups were

TABLE 3. Hematocrit, right ventricle to left ventricle plus septum (RV/LV+S) ratio, and plasma lipid peroxides at 3 and 7 wk of age in birds fed diets containing 3.5% normal fat (NF) or oxidized (OxF) fat with or without 125 ppm ethoxyquin (E)[1]

Variable	Week	Diet				Pooled SE
		NF/E	NF	OxF/E	OxF	
Hematocrit, %						
	3	28.6[b]	31.8[a]	30.8[ab]	29.2[ab]	1.2
	7	30.8	30.4	28.2	28.0	
RV/LV+S						
	3	0.22	0.24	0.21	0.21	0.02
	7	0.26[a]	0.22[ab]	0.22[ab]	0.19[b]	
Plasma lipid peroxides, ng/mL						
	3	38.0	37.5	27.0	39.0	7.1
	7	34.5	46.6	41.2	46.2	

[a,b]Means within a row with no common superscript differ significantly ($P < 0.05$).

[1]Data represent the mean ± SE of 12 observations.

TABLE 4. Reduced (GSH) and oxidized (GSSG) glutathione in the liver, duodenum and mid-ileum in birds fed diets containing 3.5% normal fat (NF) or oxidized (OxF) fat with or without 125 ppm ethoxyquin (E)[1]

Item	Diet				Pooled SE
	NF/E	NF	OxF/E	OxF	
Liver, μmol/g					
3 wk					
GSH	2.42	2.38	2.34	2.13	0.15
GSSG	0.16[a]	0.15[a]	0.16[a]	0.11[b]	0.01
7 wk					
GSH	2.76	2.36	2.72	2.65	0.16
GSSG	0.04	0.05	0.02	0.02	0.01
Duodenum, μmol/g					
3 wk					
GSH	4.03[ab]	3.66[b]	4.15[a]	3.87[ab]	0.16
GSSG	0.02	0.03	0.02	0.03	0.01
7 wk					
GSH	4.80[a]	4.14[b]	4.97[a]	4.75[a]	0.18
GSSG	0.02	0.02	0.04	0.03	0.01
Ileum, μmol/g					
3 wk					
GSH	3.50	2.82	3.65[a]	2.60[b]	0.29
GSSG	0.13	0.08	0.11	0.09	0.04
7 wk					
GSH	3.77	4.19	3.58	3.93	0.29
GSSG	0.17	0.22	0.18	0.11	0.04

[a,b]Means within a row with no common superscript differ significantly ($P < 0.05$).

[1]Data represent the mean ± SE of 12 observations.

observed at 3 or 7 wk. Cumulative mortalities (up to 7 wk) attributed to PHS were 7, 15, 7, and 3% for NF/E, NF, OxF/E, and OxF diets, respectively, and were not significantly different.

The effect of dietary treatment on GSH and GSSG in liver, duodenum, and ileal tissues is presented in Table 4. At 3 and 7 wk, there were no differences in liver GSH. Liver GSSG was lower in birds fed OxF at 3 wk than in birds on the other treatments, but there were no differences in liver GSSG at 7 wk. Duodenal GSH concentrations in birds fed OxF/E at 3 wk were higher than birds fed NF but were not different from concentrations of those fed NF/E or OxF. At 7 wk, duodenal GSH values were lower in broilers fed NF relative to the other three groups. Ileal GSH was higher in broilers fed OxF/E than in those fed OxF at 3 wk, but no differences were observed between groups at 7 wk. There were also no differences in duodenal or ileal GSSG among treatments at 3 or 7 wk.

There was no main effect of fat or fat by age interaction on tissue GSH or GSSG (data not shown) with the exception that at 7 wk, birds fed NF had higher hepatic GSSG (0.04 ± 0.01 μmol/g) than birds fed OxF (0.02 ± 0.01 μmol/g). The presence of ethoxyquin (E+) in the diet was associated with higher levels of GSH in the duodenum at 3 and 7 wk, and in the ileum at 3 wk than those of birds that did not receive ethoxyquin (E–) (Figure 1a). All tissues exhibited higher GSH levels at 7 wk than at 3 wk of age. The presence of dietary ethoxyquin had no affect on tissue GSSG (Figure 1b). Liver obtained from 7-wk-old birds exhibited lower

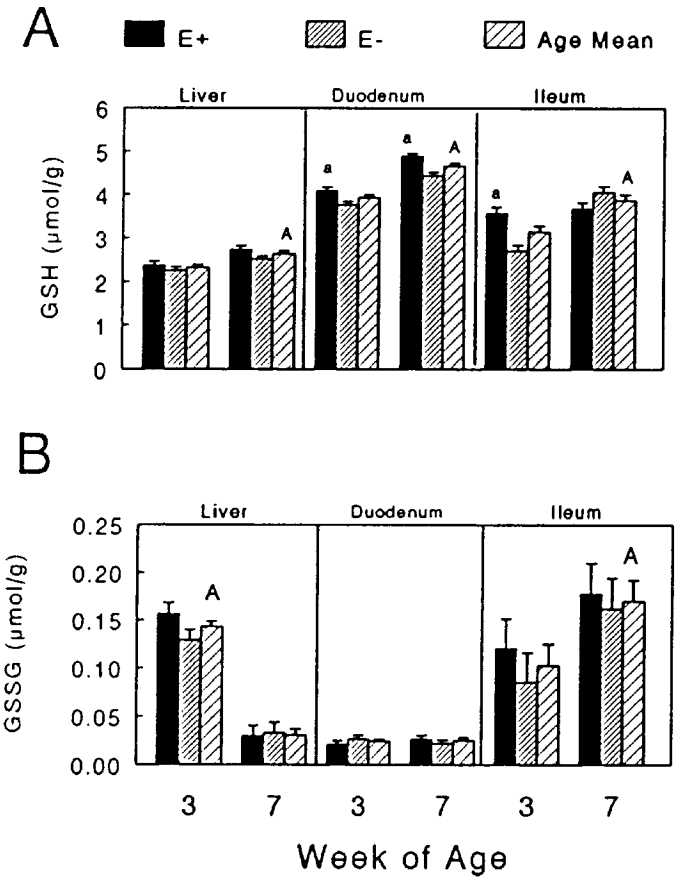

FIGURE 1. Tissue concentrations of A) reduced glutathione (GSH) and (B) oxidized glutathione (GSSG) in broilers fed diets with Ethoxyquin (E+) or without ethoxyquin (E–) at 3 and 7 wk-of-age. Values represent the mean ± SE. [a] E+ mean is higher than E– mean within each week ($P < 0.05$). [A] Mean age value is higher ($P < 0.05$).

GSSG levels than liver obtained from 3-wk-old birds, whereas ileal GSSG levels were higher at 7 wk than at 3 wk. Duodenal GSSG levels were much lower than GSSG levels in the ileum.

DISCUSSION

Birds fed diets containing ethoxyquin diets exhibited higher duodenal GSH levels at 3 and 7 wk, and higher ileal GSH at 3 wk, than birds provided diets without ethoxyquin (Figure 1a). Mechanisms by which ethoxyquin might elevate tissue GSH could be either by induction of GSH synthetic enzymes or by antioxidant sparing of GSH by lowering oxidative stress in the intestinal lumen. Sparing of antioxidant activity is often observed, as high levels of one antioxidant can overcome deleterious deficiencies in another antioxidant (Frei, 1994). Maurice *et al.* (1991) hypothesized that the intestinal mucosa of birds is subject to a higher oxidative stress than is the intestines in mammals. Thus, ethoxyquin may spare intestinal GSH by lowering the proxidant load in the intestinal lumen of broilers. Several studies also indicate that enzyme induction by ethoxyquin occurs in mammals. For example, ethoxyquin elevated hepatic thiol concentrations and induced several microsomal enzymes as well as GSH-s-transferase in mice (Wattenberg, 1980; Miranda *et al*., 1981; Cha *et al*., 1982; Kim, 1984). However, induction of GSH synthetic enzymes would require absorption of ethoxyquin and it is questionable whether significant amounts of ethoxyquin are absorbed in the broiler chicken (J. Dibner, unpublished observations). Consequently, it is not apparent whether higher duodenal GSH in birds fed ethoxyquin was by direct induction of GSH synthetic enzymes or was indirect via antioxidant sparing of GSH. Regardless of the mechanism, higher intestinal GSH levels in birds fed ethoxyquin would be beneficial to the animal in terms of the ability of GSH to conjugate toxins or combat other forms of oxidative challenges encountered by the intestines.

Liver GSH concentrations in this study and several other reports (Boebel and Baker, 1983; Chung *et al*., 1990; Murphy and King, 1990; Beers *et al.* 1992; Enkvetchakul *et al*., 1993; Spurlock and Savage, 1993; Enkvetchakul and Bottje, 1995) are well below GSH values typically reported in mammalian liver (e.g., Meister, 1984). Also, duodenal and ileal GSH levels were higher than levels in the liver (Figure 1). This latter observation also differs from those in mammals, in which the liver generally contains the highest levels of GSH in the body (Meister, 1984). The liver in mammals provides GSH to other tissues via interorgan circulation (Anderson *et al.* 1980). If a similar interorgan circulation exists in birds, the intestines would then represent a major sink for GSH due to its size and high activity of γ-glutamyl transpeptidase activity, which is responsible for transport of GSH into cells. Thus, the increases in intestinal GSH observed in birds fed ethoxyquin would be of even greater importance, as this could allow exported liver GSH to be utilized by tissues other than the intestines.

Concentrations of GSSG were higher in the ileum than in the duodenum and were higher in ileal tissue obtained from 7-wk-old broilers than in ileal tissue obtained from 3-wk-old broilers (Figure 1). Cellular GSSG levels are normally kept low by the action of GSH reductase (Meister, 1984), as high GSSG levels are toxic to the cell (Reed, 1990). Thus, higher GSSG levels in the ileum, in comparison to the duodenum, could indicate a general pervasive oxidative stress that increased with age. On the other hand, lower liver GSSG at 7 wk than at 3 wk might reflect an enhanced ability to metabolize GSSG by heightened GSH reductase activity in the liver of older birds.

The high levels of dietary peroxides were intentionally used in this study in an attempt to increase oxidative stress and the incidence of PHS as oxidative stress may play a role in the pathophysiology of PHS (Bottje *et al*., 1995; Bottje and Wideman, 1995). Growth suppression is well known to reduce the incidence of PHS, however. Slower growth rates could lower oxidative stress by decreasing the amount of free radicals generated during metabolism (Bottje and Wideman, 1995). Thus, the slower growth rate associated with feeding oxidized fat may have counteracted the negative effects of added oxidative load represented by feeding high levels of oxidized fat and account for the lack of difference in PHS mortality between dietary treatments in this study.

Growth suppression from oxidized fat has been well documented in several different animal species (Nakamura *et al*., 1972; Reddy and Tappel, 1974; Inoue *et al*., 1984; Shermer and Calabotta, 1985; Cabel *et al*., 1988). The presence of high amounts of oxidized fat in the diet raises the levels of aldehydes and other oxidized metabolites that are toxic to animals and can not be reduced by ethoxyquin. Growth suppression by oxidized fat was clearly shown by Cabel *et al.* (1988), who demonstrated that weight gain was unaffected when broilers were fed diets containing a peroxide level of 4 meq or less/kg of feed, but growth rate was impaired in broilers fed diets containing 7 meq peroxide/kg. Furthermore, the growth rate impairment observed by feeding 7 meq peroxide/kg could be ameliorated by feeding 125 but not 62.5 ppm ethoxyquin. In the present study, peroxide levels were 11.6 and 32.4 meq/kg feed in birds fed OxF/E starter and grower diets and were 6 to 7 meq less than that provided to birds assigned to the OxF diet. Thus, the level of peroxides present in the OxF diets used in this study exceeded the protective efficacy of ethoxyquin demonstrated by Cabel *et al.* (1988), and accounts for the lack of difference in growth rates in birds fed OxF/E and OxF diets (Table 2).

In summary, the slow growth rate observed in birds fed high amounts of oxidized fat (greater than 17 meq peroxide/kg feed) may have offset any potentiation of PHS mortality due to increased oxidative stress. Growth

rate during the starter period was higher in birds fed normal fat plus ethoxyquin than in those receiving normal fat alone. Ethoxyquin elevated intestinal tissue GSH but additional studies are required to determine whether this increase was due to active induction of GSH synthetic enzymes or a passive sparing of GSH by decreasing the oxidative load on the cells.

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