Effects of Long-Term Vitamin E and Butylated Hydroxytoluene Supplemented Diets on Murine Intestinal and Hepatic Antioxidant Enzyme Activities

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ABSTRACT : The present study was designed to determine long-term feeding effects of vitamin E and BHT (butylated hydroxytoluene) on serum biochemical profiles, organ weight, and intestinal and hepatic antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), and glutathione-S-transferase (GST) in ICR mice. Four wk old ICR mice (n=8 per group) were fed the diets supplemented with vitamin E (I ; 0.03% and II ; 0.3%) and BHT (I ; 0.05% and II ; 0.5%) for 12 months. Feeding the diets containing vitamin E and BHT had no effects on growth and serum biochemical profiles. However, feeding the diets supplemented with 0.5% BHT for 12 months significantly increased liver weight of the mice. In the small intestine, there were no effects of vitamin E or BHT on SOD and GSH-PX activities in the mucosa. However, the activity of intestinal GST of the mice that received 0.5% BHT was almost twice as high as that of control mice. In the liver, the activity of SOD was not affected by feeding antioxidants for 12months, whereas GSH-PX activity was significantly increased in mice that received the diets containing BHT (0.05%, 0.5%) and vitamin E (0.03%, 0.3%). In addition, supplementation of 0.5% BHT markedly enhanced hepatic GST activity compared with other groups. Enhanced activity of GSH-PX in response to feeding vitamin E or BHT might aid hepatic enzymes to eliminate active oxygen in organs from mice. However, we could not exclude the possibility of increased lipid peroxidation by high dosage of BHT supplementation. More detailed study is necessary for assessment of preventive or toxicological effects of high dosage of BHT supplementation. *(Asian-Aus. J. Anim. Sci. 1999. Vol. 12, No. 6 : 932-938)*

Key Words : Long-Term, Vitamin E, BHT, Antioxidant Enzymes, Intestine, Liver

INTRODUCTION

Antioxidants are widely used as feed additives in the animal feed industry due to their potential beneficial effects, including preservation of feed and improvement of animal health. Especially, a diet containing polyunsaturated fatty acid is particularly susceptible to changes in color, taste, and odor under oxidative degradation when it is exposed to air (Sevanian and Hochstein, 1985). Antioxidants such as vitamin E, BHT (butylated hydroxytoluene), BHA (butylated hydroxyanisole), and exthoxyquine are used as feed additives for the preservation of diets containing high fat. Among these antioxidants, vitamin E is well recognized not only as a natural antioxidant for prevention of biological oxidation but also as an essential nutrient for increasing cell mediated immunity (Porta et al., 1980; Kline and Sanders, 1991). BHT, one of the widely used synthetic phenolic antioxidants, was found to provide significant protection against biological oxidation and cancer (Slaga, 1995). This phenolic compound also has been known to affect the detoxification mechanism of xenobiotics (Nijhoff and Peters, 1992). These antioxidants are thought to act as biological antioxidants by protecting polyunsaturated lipids against peroxidative stress, and subsequently maintaining body homeostasis during aging process. However, several deleterious effects of BHT such as carcinogenesis have been reported when animals are exposed to high doses (Malkinson and Beer, 1984).

Although the functions of these antioxidants have been well documented, the effects of long term feeding and dietary levels of antioxidants on animal health have not been extensively investigated. Especially, the long term effect of vitamin E or BHT on antioxidant efficacy or toxicity in specific organs, including the gut, is relatively ignored. Therefore, we fed two most widely used antioxidants (vitamin E and BHT) at two levels (moderate and high) for 12 months to mice to test the effect of antioxidants on serum biochemical profiles and activities of antioxidant enzymes in the small intestine and liver.

MATERALS AND METHODS

Animal diets and harvesting tissues

Forty male ICR mice (4 wk old, average BW: 18.21±2.16 g) were randomly allotted to the diets of control, 0.03% vitamin E (Vit E I), 0.3% vitamin E (Vit E II), 0.05% BHT (BHT I), and 0.5% BHT (BHT II). All groups of mice were maintained under a

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barrier system in regulated temperature (23±2℃), humidity (50±10%), and light/dark cycle (light on 07:00-19:00). All mice were fed experimental diets *ad libitum* and had free access to water for entire trial.

The composition of basal diet (commercial chow) is presented in table 1. The vitamin E (Merck, *α*-tocopherol acetate) and BHT (Sigma, butylated hydroxytoluene) were added to the corn-soybean basal diet according to the experimental design. Feed intake and individual body weight were recorded weekly. At the end of the feeding trial of 12 months, eight mice per group were deprived of feed overnight and sacrificed with ether, and whole blood was collected by heart puncture. Serum samples were harvested by 10 min centrifugation at 3,000 rpm and kept at -70℃ until use. Immediately after collection of blood from mice, the liver, spleen, kidney, and adrenal gland were separated and weighed. The small intestine and liver were stored at 70℃ to measure specific activities of antioxidant enzymes.

Table 1. Formula and chemical composition of control diet[a]

Items	Percentages
Ingredients %, as fed basis	
Corn, yellow	40.1
Fish meal	3.0
Alfalfa meal	3.0
Wheat meal	5.2
Wheat germ	7.6
Soybean meal	16.6
Corn gluten	3.0
Dried yeast	2.0
Full fat soybean	15.0
Cl_2-choline, 50%	0.06
Methionine, 50%	0.28
Biotin	0.06
Vitamins & minerals[b]	0.4
Limestone	0.78
$Ca_3(PO_4)_2$	2.42
NaCl	0.5

[a] Analyzed composition of diet (as fed basis): 24.9% C-protein, 5.85% C-fiber, 4.12% C-fat, 7.86% C-ash, 48.1% NFE, 1.40% Ca, and 0.76% P.

[b] Vitamin & mineral provided the following per kg diets: vit A 10,000 IU, vit D_3 2,000 IU, vit E 50 mg, vit K_3 2 mg, cal-pantothenate 10 mg, vit B_2 3 mg, niacin 20 mg, vit B_{12} 30 mg, folic acid 0.4 mg, vit B_1 2 mg, vit B_6 2 mg, Fe 60 mg, Zn 80 mg, I 12 mg, Co 2.4 mg, and Se 0.15 mg.

Serum biochemical components

A clinical chemical auto analyzer (ABBOT, VP) was employed for the determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), glucose, creatinine, and cholesterol in the serum.

Specific activities of antioxidant enzymes

In order to harvest cytosol from tissues of intestinal mucosa and the liver, crude homogenized tissues were centrifuged at 10,000×g and resulting supernatant was centrifuged at 105,000×g in a Centrikon T-2080 ultracentrifuge. The supernatant (cytosol) was frozen with liquid nitrogen and stored at -70℃ until further assay. Protein was assayed by BCA method (Pierce Assay) using an ELISA (Molecular Devices). The activity of superoxide dismutase (SOD) in the cytosol fractions was determined using xanthine and xanthine oxidase system for production of superoxide radical and subsequent measurement of cytochrome c as a scavenger of the radicals (Fridovich, 1974). The SOD activity was expressed as units/mg of protein, where one unit of activity is the amount of enzyme required to inhibit the rate of reduction of cytochrome c by 50%. Glutathione peroxidase (GSH-PX) was measured at 37℃ in the cytosol using cumen hydroperoxide as a substrate (Tappel, 1978). The GSH-PX coupled the reduction of cumen hydroperoxide to the oxidation of NADPH by gluthathione reductase and concomitant oxidation is monitored by spectrophotometer with the decrease in absorbance at 340 nm. One unit of GSH-PX is expressed as the amount of GSH-PX needed to oxidize 1 mol of NADPH per min. Cytosolic glutathione-S-transferase (GST) was determined using CDNB as a substrate and monitored increase in absorbance at 340 nm (Habig, 1974). One unit of activity was expressed as the amount of enzyme catalyzing the conjugated CDNB per min.

Statistical analysis

Data from antioxidant associated differences were analyzed by ANOVA procedures appropriate for completely randomized designs. Mean differences among different groups were evaluated by the Tukey test at $p<0.05$.

RESULTS

Changes in body weight, organ weight, and serum biochemical profiles

The effects of dietary additions of vitamin E and BHT on body weight and organ weight are presented in figure 1 and table 2, respectively. Body weight increased continually until the age of 12 months in all treatment groups, but the supplementation of vitamin E (0.03%, 0.3%) and BHT (0.05%, 0.5%) did not affect body weight or gain (figure 1). Weights of the brain, spleen, and kidney were not altered by dietary supplementation. However, weight of the liver was significantly heavier in the group of mice fed 0.5%

BHT compared with other groups. As shown in table 3, dietary supplementation of vitamin E and BHT at both levels had no significant effect on AST, ALT, BUN, glucose, creatinine, and cholesterol levels in the serum.

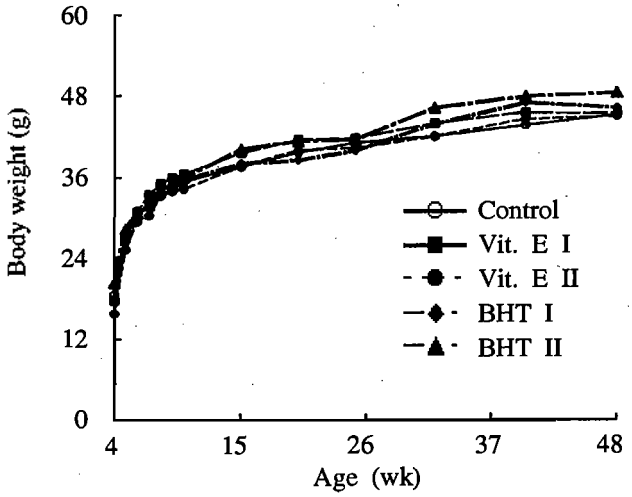

Figure 1. Changes in body weight in ICR mice fed the diets supplemented with vitamin E and BHT for 12 months: control, Vit E I (0.03%), Vit E II (0.3%), BHT I (0.05%), and BHT II (0.5%)

Specific activities of antioxidant enzymes in the small intestine and liver

Intestinal SOD, GSH-PX, and GST activities in the cytosol of mucosae are shown in figure 2 (A, B, C). No effect of vitamin E or BHT on cytosolic SOD (figure 2, A) and GSH-PX (figure 2, B) activities in the small intestine were observed. However, GST activity (figure 2, C) was markedly enhanced in the mice fed diets enriched with 0.05% BHT and 0.5% BHT. Especially the GST activity in those fed the 0.5% BHT diet was almost twice as high as the mice fed the control diet.

Hepatic SOD, GSH-PX, and GST activities from mice fed diets supplemented with vitamin E and BHT are shown in figure 2 (D, E, F). The specific activity of cytosolic SOD (figure 2, D) was not affected by dietary addition of antioxidants. However, significantly increased GSH-PX activity was observed in response to dietary addition of vitamin E and BHT compared with control; there was also a significant difference in GSH-PX activity between vitamin E and BHT. Significantly elevated GST was observed in the mice fed 0.5% BHT supplemented diet. Dietary supplementation of BHT had a more profound effect on increasing GSH-PX and GST activities than vitamin E.

Table 2. Changes in organ weight in ICR mice fed the diets supplemented with vitamin E and BHT for 12 months

Group	Liver (g)	Spleen (mg)	Kidney (mg)	Brain (mg)
Control	1.48±0.26[a]	95±27	505±34	657±115
Vit E I	1.78±0.30[a]	80±22	540±25	832±135
Vit E II	1.77±0.32[a]	117±33	525±25	803± 62
BHT I	1.64±0.46[a]	93±35	512±29	741±238
BHT II	2.47±0.23[b]	112±20	504±15	722± 83

[a,b,c] Means within same column with different superscripts differ significantly at $p<0.05$. Values are Mean±SD. Vit E I (0.03%), Vit E II (0.3%), BHT I (0.05%), and BHT II (0.5%).

DISCUSSION

It was demonstrated that a long duration of feeding vitamin E seemed to increase life span in experimental animals (Porta et al., 1980). A survey of the pertinent literature (Panczenko-Kresowska and Ziemlanski, 1987; Siman and Eriksson, 1996) and the present result suggest that diets supplemented with high levels of vitamin E (up to 0.4%) and BHT (up to 1.0%) do not affect body weight in mice and rats. However, similar to our result, BHT-supplemented 0.2% (Yamamoto et al., 1995), 0.5% and 1.0% (Siman and Erilsson, 1996) markedly enhanced liver weight compared with control mice and rats. A general hyperplasia of the liver caused by BHT feeding had been shown to occur without any histological changes (Johnson and Hewgil, 1961). More detailed study is necessary to understand why this increase in liver weight occurs.

The effect of antioxidants on blood biochemical components seemed to vary according to dietary

Table 3. Changes in serum biochemical profiles in ICR mice fed the diets supplemented with vitamin E and BHT for 12 months

Group	ALT (IU/l)	AST (IU/l)	BUN (mg/l)	Glucose (mg/l)	Creatinine (mg/l)	Cholesterol (mg/l)
Control	49.6±12.1	171.3±32.1	4.14±0.89	15.81±3.22	0.065±0.006	14.1±0.7
Vit E I	52.3±14.0	164.0±40.1	3.91±1.45	16.04±4.74	0.072±0.013	12.9±1.8
Vit E II	49.0±15.8	152.1±37.8	3.57±0.89	13.31±3.23	0.073±0.003	11.9±2.9
BHT I	54.8±11.2	148.9±33.2	4.12±0.12	13.52±5.37	0.066±0.006	13.5±0.6
BHT II	54.0±10.9	139.5±39.9	4.45±0.99	14.81±4.12	0.072±0.006	13.4±2.7

Values are Mean±SD. Vit E I (0.03%), Vit E II (0.3%), BHT I (0.05%), and BHT II (0.5%).

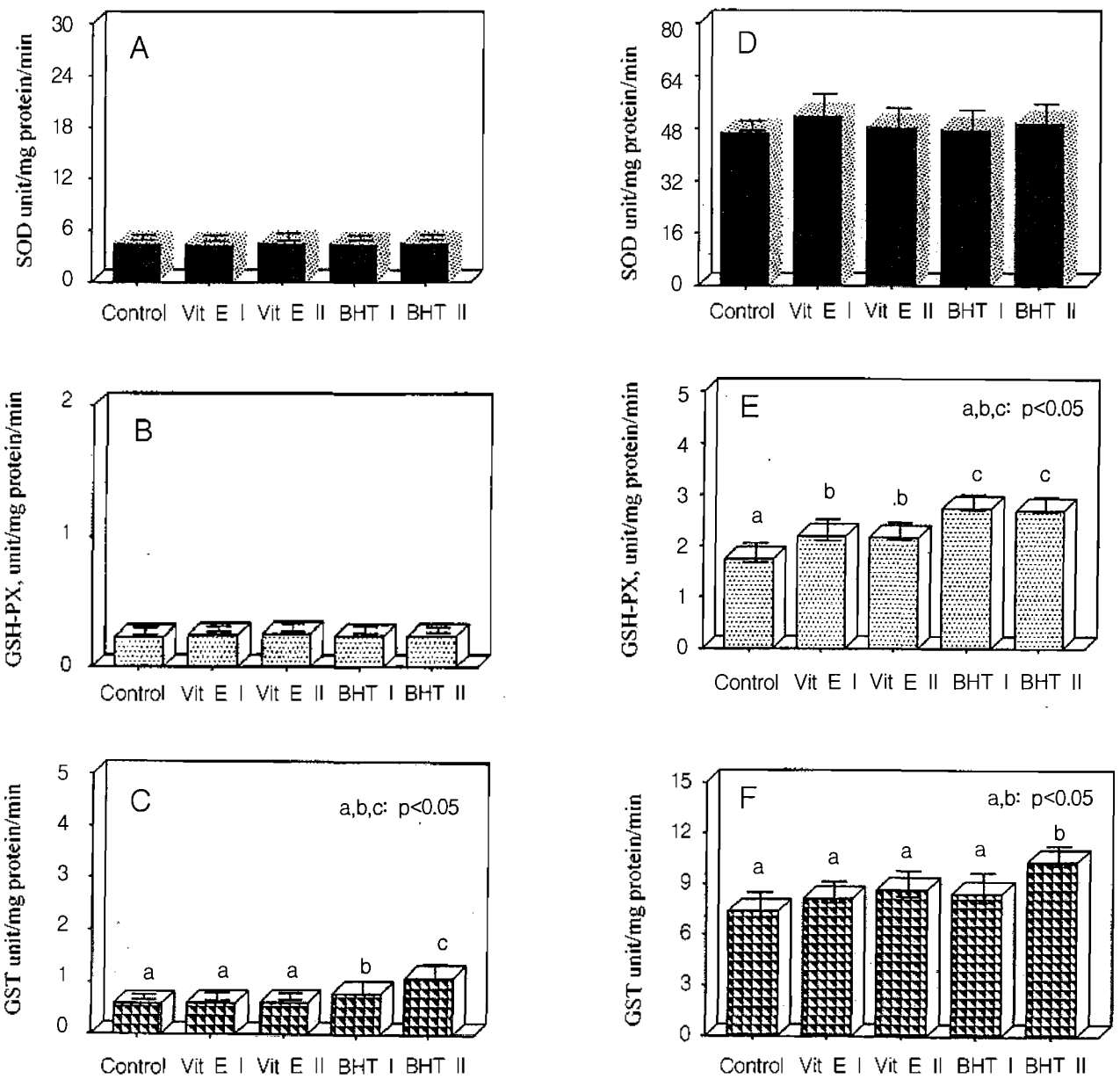

Figure 2. Changes in specific activities of intestinal and hepatic SOD (A, D), GSH-PX (B, E), and GST (D, F) in ICR mice fed the diets supplemented with vitamin E and BHT for 12 months
The values represent the mean ± SD (n=8); control, Vit E I (0.03%), Vit E II (0.3%), BHT I (0.05%), and BHT II (0.5%). [a,b,c] Means with different superscripts differ significantly at $p<0.05$.

components, feeding period, and age. Especially, since the preventive effect of antioxidant against atherosclerosis was known (Bjorkem et al., 1991), the relationship between antioxidants and cholesterol level was intensively investigated. However, the effect of antioxidants on blood cholesterol level is much less clear. Many studies reported that vitamin E did not affect blood cholesterol in human and rats (Meydani et al., 1994; Panczen-Kresowska and Ziemlanski, 1987). However, it was reported that dietary addition of 0.5% (Konneh et al., 1995) and 2% vitamin E (Munday et al., 1998) decreased blood cholesterol level in rats and mice. In contrast, BHT increased cholesterol and triglyceride levels in rabbit (Bjorkhem et al., 1991). Several studies reported that serum glucose concentration was higher in vitamin E supplemented calves (Reddy et al., 1987), although the reason for this change is not apparent.

AST, ALT, and LDH are intracellular enzymes involved in amino acid or carbohydrate metabolism. These are present in high concentrations in the liver, muscle, and brain. Elevated concentrations of these

enzymes in the blood indicate liver necrosis or disease (Murray et al., 1990). It is sometimes noticeable that elevated serum LDH activity has been reported in vitamin E deficiency animals partly because of enhanced liver damage by lipid peroxidation (Reddy et al., 1987); we did not observe any difference in serum AST or ALT in mice fed either high dose of vitamin E or BHT. The cause of increased liver weight in 0.5% BHT groups was not explainable with serum biochemical indicators in our data. Yamamoto et al. (1995) also did not observe any difference in AST and ALT in the blood when rats were fed with diet containing 0.2% BHT, but it was reported that long term feeding of diets supplemented with a high level of BHT induced toxicity in the liver, lung, and blood (Kahl and Kappus, 1993). In general, vitamin E can be used in high doses without the occurrence of adverse effects (Kahl and Kappus, 1993).

Among various organs, the small intestine provides the first membrane barrier between the external and internal environment, and subsequently there are many defense and adaptation mechanisms against oxidants and xenobiotics (Nijhoff and Peters, 1992). Information on the small intestine regarding an anti-oxidant defense system according to dietary antioxidant intake are very limited. Sometimes, an intracellular antioxidant system in the intestine is more remarkable than that of the liver under ingestion of chemicals (Albrecht et al., 1992; Nijhoff and Peters, 1992). From our observations, intestinal SOD and GSH-PX were not influenced by dietary vitamin E and BHT supplementation. However, intestinal GST activity was markedly elevated with dietary intake of 0.5% BHT. The capability of mucosal cells to detoxify these substances is essential for protection against xenobiotics. Therefore, the elevated GST in the intestine with BHT intake may play an important role in the biotransformation of chemical substances (de Waziers et al., 1988).

The liver plays the central role in coordinating the metabolism of ingested nutrients and xenobiotics. The relationship between antioxidant enzyme activities and vitamin E supplementation is still controversial according to studies with rodents. The addition of vitamin E (1,000 mM) and BHT (1,000 mM) to rat kidney cells significantly increased SOD and catalase activities (Guyton et al., 1995). Khann et al. (1992) reported that vitamin E and BHA (butylated hydroxyanisole) significantly enhanced activity of glutathione reductase in mice liver. Also, other studies reported that vitamin E (McIntosh et al., 1993) or BHA (Nijhoff and Peters, 1992) markedly increased hepatic antioxidant enzymes or GST activities. However, several studies indicated that vitamin E did not alter antioxidant enzyme activities (Shaman et al., 1993; Li et al., 1996). The protective effect of vitamin E on lipid peroxidation may not be due to alteration of antioxidant enzymes but may be mainly mediated through its chain-breaking antioxidant activity (Mantha et al., 1993). Our previous study (Jang et al., 1997) with mice indicated that dietary supplementation of vitamin E for 12 wks did not affect activities of antioxidant enzymes such as SOD and GSH-PX. The lack of agreement among various studies including our result on antioxidant enzymes is likely due to differences in dosage, age, diet composition, periods of experiment, even animal strain and gender (Egaas et al., 1995). Our data indicating increased hepatic GSH-PX activity in response to dietary vitamin E (0.03%, 0.3%) and BHT (0.05%, 0.5%) for 12 months suggest that antioxidant enzymes may have a role in reducing hepatic peroxidation and subsequent self-protective mechanism from repairing oxidized fatty acid from lipid membrane. Enhanced activity of one or more of the antioxidant enzymes may reduce lipid peroxidation (Xia et al., 1995).

Compared with other antioxidant enzymes, increased GST activities give us interesting results. The induction of GST in the small intestine and liver in 0.5% BHT group may be considered to have increased the protection mechanism against biological oxidation in these organs. It was recently demonstrated that BHT did not have any adverse effects on either reproductive or neurobehavioral parameters when given at a dose of 0.4% in the diet (Tanaka et al., 1993). However, care must be taken in interpretation of increased GST activity in mice that received 0.5% BHT. One feasible factor that may affect the increased activity of GST, drug-metabolizing phase II enzyme, is a concomitant increase in the free radical formation in mice fed high dosage of BHT. Significantly increased GST activity in the intestine and liver from mice fed 0.5% BHT has an important role in defense against oxidation damage to help repair oxidized fatty acid. Yamamoto et al. (1995) reported that 0.2% BHT feeding increased production of lipid peroxidation in the liver. It is therefore likely that an increase in GST activity is partly due to increase in lipid peroxidation by dietary BHT supplementation. The animal receiving a high dose of BHT for long period is needed to evaluate possible toxicological effects, although hepatic antioxidant activity of GSH-PX is increased by dietary addition of BHT. Studies on the exact mechanism of BHT-induced GST activity may be of importance for assessment of preventive or toxicological effect of BHT.

Consideration must be given to the dietary regime in our experiment. The basal diet used for our experiment already contains 0.005% of vitamin E. This amount (50 mg/kg) is considered to be the minimum requirement for maintaining an optimal immune system and physiological status (Bendich et al., 1986). Dietary

supplementation of 0.02% BHT is allowed in domestic animals (Maeng, 1994). From our data, high dosage of antioxidants gave rise to increased activities of antioxidant enzymes in mice compared with the mice fed optimal levels of antioxidants.

It is concluded that intestinal and hepatic GST and hepatic GSH-PX activities were markedly enhanced in mice fed diets supplemented with high dosages of vitamin E or BHT. The elevated hepatic antioxidant enzymes may be an adaptive response to protect tissue damage from free radicals, although it is not clear whether increased activities of antioxidant enzymes especially in the mice fed a high dosage of BHT are due to increased lipid perioxidation or not.

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