



EFFECTS OF BUTYLATED HYDROXYTOLUENE ON BLOOD LIVER ENZYMES AND LIVER GLUTATHIONE AND GLUTATHIONE-DEPENDENT ENZYMES IN RATS

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Summary

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This study was aimed to detect the effect of butylated hydroxytoluene (BHT) on the liver glutathione level and glutathione-dependent enzyme activities in female Wistar albino rats. BHT was administered by oral gavage at a dose of 250 mg/kg (Group I) and 500 mg/kg (Group II) for 28 days, 1000 mg/kg (Group III) and 1500 mg/kg (Group IV) for 4 days. The serum ALT, AST and LDH activities were measured on an autoanalyzer, and liver glutathione (GSH), glutathione peroxidase (GPx), glutathione S-transferase (GST), and glutathione reductase (GR) activities were analysed with commercial ELISA kits. The ALT activity was significantly higher in Groups III and IV ($P < 0.05$ and $P < 0.001$, respectively) compared to the control group. Blood AST and LDH activities were significantly increased in Group IV ($P < 0.05$). The GSH, GPx, GST and GR in the liver tissue were determined to be statistically low in Groups II, III and IV ($P < 0.001$) in comparison with control group. In microscopic examination, BHT caused histopathological changes in the rat liver tissue in Groups II, III and IV depending on the dose and duration of exposure. It can be concluded that BHT plays a role in producing liver damage in rats with depressed hepatic antioxidant defense. The hepatotoxic response seemed to be dose- and time-dependent.

Key words: antioxidant enzymes, butylated hydroxytoluene, glutathione, histopathology, liver

INTRODUCTION

Butylated hydroxytoluene (2,6-di-tert-butyl-p-kresol or 2,6-di-tert-butyl-4-methyl phenol; BHT) is a synthetically prepared phenolic antioxidant, which is mainly used in food containing solid and liquid

fat, packing material and cosmetic products. Fat and fat-containing foods are susceptible to fermentation and other oxidation reactions. BHT acts as radical scavenger against lipid oxidation resulting of

free radical chain reaction and thereby protects foods from deterioration (Lanigan & Yamarik, 2002). BHT is generally recognised as a safe food additive (E 321). The Joint Expert Committee on Food Additives of the FAO/WHO approved an acceptable daily intake for man of 0.5 mg BHT/kg body weight. It is rapidly absorbed from the gastrointestinal tract and distributed to liver and body fat (Panicker *et al.*, 2014).

BHT is primarily metabolised in the liver microsomes. Rat liver BHT is metabolised to a variety of sulfate and glucuronic acid conjugates eventually excreted in the urine and faeces and to some extent through bile (Panicker *et al.*, 2014). Cytochrome P450 mediated metabolism in liver causes the formation of a reactive and electrophilic metabolite BHT quinone methide (BHT-QM; 2,6-di-tert-butyl-4-methylenecyclohexa-2,5-dienone), an intermediate metabolite of BHT which can covalently bind to various cellular nucleophiles, especially those containing sulfhydryl groups such as cysteine and glutathione which consequently leads to acute hepatotoxicity (Devi *et al.*, 2003). Some investigations on the mechanism of BHT-induced plasma transaminase activity were used to indicate toxicity (Nakagawa, 1987).

The endogenous glutathione (GSH), synthesised mainly in the liver, plays an important role in the cell defense system. GSH, being a thiol compound, acts in cells as an antioxidant. GSH, as a cofactor of glutathione peroxidase (GPx), participates in the reduction of peroxides with concomitant formation of oxidised glutathione disulfide (GSSG). At normal physiological conditions GSSG is reduced to GSH by glutathione reductase (GR) at the expense of reduced nicotinamide adenine dinucleotide phosphate (NADPH),

thereby forming a redox cycle (Parke & Piotrowski, 1996). The glutathione peroxidase/glutathione reductase redox cycle is responsible for the maintenance of proper GSH concentration (Dringer, 2000). GSH is also involved in detoxication of many xenobiotics through the formation of S-conjugates with toxic metabolites in the second phase of biotransformation. GSH forms S-conjugates also with products of lipid peroxidation. The reaction of S-conjugation can be significantly accelerated by glutathione S-transferase (GST) (Jurczuk *et al.*, 2006).

Butylated hydroxytoluene has been subjected to extensive toxicological studies in various animals. The LD₅₀ value of BHT in rats when administered orally is >2930 mg/kg. At high doses it is known to cause haemorrhagic death because of the inhibition of hepatic prothrombin synthesis. BHT has been shown to induce reversible mixed function oxidases and liver enlargement in rats and peliosis, hepatocellular vacuolation, degeneration and necrosis in the liver of mice and found to increase the mitotic activity of liver cells in rats (Devi *et al.*, 2003).

BHT is used to treat herpes in humans and is found to be effective against a wide variety of lipid coated viruses like Newcastle disease virus. BHT can act both as promoter and antipromotor of carcinogenesis (Panicker *et al.*, 2014). Despite the antioxidant and antiviral effects, many questions about the use of BHT remain unanswered. Therefore, the present study was carried out to evaluate the toxic effects of BHT in rat liver.

MATERIALS AND METHODS

Animals

Fourty seven female Wistar Albino rats (200–250 g on average) were used in the

study. They were housed in the cages on a 12 h light-dark cycle at 22 ± 2 °C. They were maintained on food pellets and drinking water *ad libitum*. The study was approved by the Animal Use Ethics Commission (31.03.2014) of Yüzüncü Yıl University.

Experimental design

The rats were randomly divided into five groups as control (n=7) and BHT groups (n=10). The rats in the control group were given only corn oil.

BHT (Sigma-B1378, E321) was dissolved in corn oil and administered by oral gavage to the different treatment groups as followed: Group I (250 mg/kg for 28 days), Group II (500 mg/kg for 28 days), Group III (1000 mg/kg for 4 days) and Group IV (1500 mg/kg for 4 days) (Powell *et al.*, 1986).

Sample collection and analysis

At the end of the trial period, intraperitoneal 75 mg/kg ketamine HCl was injected, and the blood was collected by heart puncture. Then, the collected blood was centrifuged at +4 °C, 3000 rpm for 5 min to obtain serum. ALT, AST and LDH activities in the extracted serum were measured on an autoanalyser (Roche/Hitachi) using commercial kits.

The rats were immediately sacrificed by bloodless method under ketamine anaesthesia, and the liver was excised. Samples of liver tissue were homogenised in cold 0.1 mM phosphate buffer (pH 7.4), and centrifuged at 10,000 rpm at 4 °C for 15 min. The GSH level and GPx, GR, GST activities in the collected supernatants were measured by ELISA (Zenyth 200 rt) using commercial kits (Cayman Chemical Company, Ann Arbor, MI, 2014).

Histopathological analysis

For histopathological evaluation, the liver tissue samples were fixed in 10% formalin solution for 48 h, and then washed in tap water for 8 h. During the routine tissue control stage, they were treated with alcohol (70°, 80°, 90°, 96°, 100°) and xylene, embedded in paraffin. Slides of 4 µm thickness were cut from each block, stained with haematoxylin-eosin (H&E), investigated under light microscope and the relevant areas were photographed (Luna, 1968; Taylor & Cote, 1994).

Statistical analysis

The Kolmogorov-Smirnov test was used to analyse the normal distribution of continuous data. For the variables showing normal distribution, one way analysis of variation (one-way ANOVA) was used for the comparison of the groups. The level of significance was set up to $P < 0.05$, $P < 0.001$. All data were analysed by using SPSS 20.0 software.

RESULTS

Biochemical results

Blood ALT activity was significantly increased in in Groups III and IV ($P < 0.05$ and $P < 0.001$, respectively) with regard to the control group, while no statistically significant change was observed between the other two groups and the control group. The AST and LDH activities were found to be significantly increased in Group IV ($P < 0.05$) compared to all other groups (Table 1). The liver tissue GSH level and GPx, GST, GR activities were significantly decreased in Groups II, III and IV when compared to the control group ($P < 0.001$), and no significant difference was detected between Group I and the control group (Table 2).

Table 1. Effect of butylated hydroxytoluene on the activities of ALT, AST and LDH in rat serum. Values are presented as mean \pm SD (n=7 for controls, n=10 for all treatment groups)

Groups	ALT (U/L)	AST (U/L)	LDH (U/L)
Control	36.62 \pm 2.10 ^a	126.18 \pm 13.79 ^a	1041.57 \pm 150.48 ^a
Group I 250 mg/kg; 28 days	39.12 \pm 2.14 ^a	160.55 \pm 12.24 ^{ab}	1156.57 \pm 185.79 ^{ab}
Group II 500 mg/kg; 28 days	61.47 \pm 6.70 ^{ab}	176.95 \pm 19.49 ^{ab}	1212.28 \pm 107.87 ^{ab}
Group III 1000 mg/kg; 4 days	82.62 \pm 3.77 ^{bc*}	218.20 \pm 11.80 ^{ab}	1221.00 \pm 220.09 ^{ab}
Group IV 1500 mg/kg; 4 days	98.51 \pm 13.84 ^{c**}	306.24 \pm 90.74 ^{b*}	2280.85 \pm 543.78 ^{b*}

The difference among the group averages with different letters in the same column is statistically significant, *P<0.05, ** P<0.001.

Table 2. Effect of butylated hydroxytoluene on the level of rat liver glutathione (GSH), glutathione peroxidase (GPx), glutathione S-transferase (GST), and glutathione reductase (GR). Values are presented as mean \pm SD (n=7 for controls, n=10 for all treatment groups)

Groups	GSH (nmol/min/mg)	GPx (nmol/min/mg)	GST (nmol/min/mg)	GR (nmol/min/mg)
Control	4.43 \pm 0.04 ^a	210.00 \pm 3.40 ^a	289.85 \pm 2.63 ^a	198.00 \pm 1.91 ^a
Group I 250 mg/kg; 28 days	4.27 \pm 0.03 ^a	202.71 \pm 1.86 ^{ab}	273.71 \pm 6.78 ^{ab}	190.42 \pm 2.51 ^a
Group II 500 mg/kg; 28 days	1.72 \pm 0.06 ^{b**}	193.85 \pm 2.14 ^{b**}	260.85 \pm 6.13 ^{b**}	178.14 \pm 1.95 ^{b**}
Group III 1000 mg/kg; 4 days	1.01 \pm 0.05 ^{c**}	159.28 \pm 1.12 ^{c**}	197.57 \pm 4.05 ^{c**}	173.14 \pm 1.69 ^{b**}
Group IV 1500 mg/kg; 4 days	0.45 \pm 0.01 ^{d**}	81.57 \pm 2.47 ^{d**}	143.00 \pm 6.06 ^{d**}	130.71 \pm 1.92 ^{c**}

The difference among the group averages with different letters in the same column is statistically significant, *P<0.05, ** P<0.001.

Histopathological results

The liver tissue was normal in the control group (Fig. 1A) and Group I (Fig. 1B); hepatic congestion and mild degeneration were detected in central hepatocytes in Group II (Fig. 1C); congestion, degeneration, many necrotic fields and haemorrhagic foci in central hepatocytes were observed in Group III (Fig. 1D). Around

the necrotic foci and in the portal region in Group IV (Fig. 1E,F), many necrotic, haemorrhagic fields in the central region and lympho-plasmocytic cell infiltration were detected. Although the histopathological findings in the Groups III and IV were similar, necrotic haemorrhagic changes in Group IV were more severe.

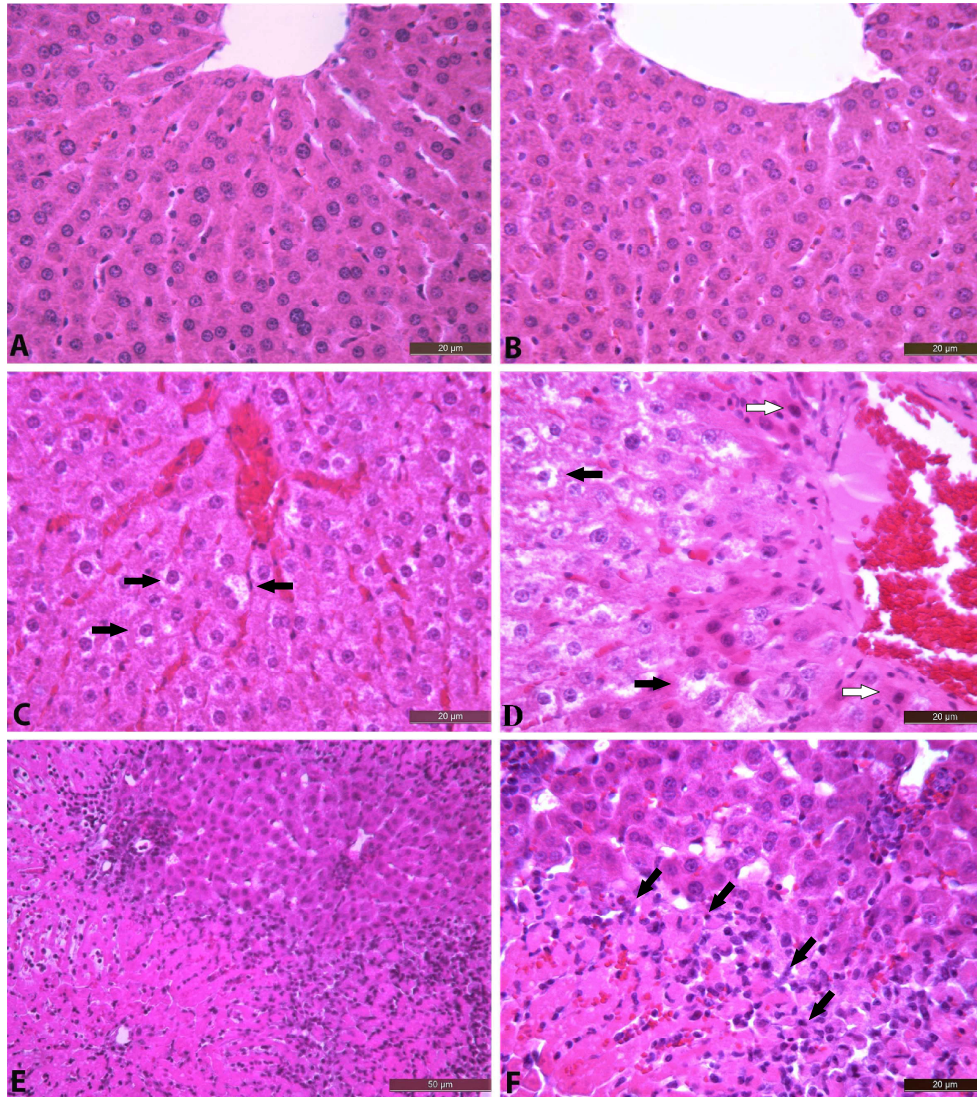


Fig. 1. Histopathological structure of the liver tissue of rats (H&E staining). **A.** Control group (bar: 20 µm). **B.** Group I. The liver tissue structure is normal (bar: 20 µm). **C.** Group II. Hepatic congestion in the liver tissue and degeneration in the hepatocytes (bar: 20 µm). **D.** Group III. Congestion in the liver tissue, degeneration in the hepatocytes (black arrows), a number of necrotic areas and haemorrhagic foci (white arrows), (bar: 20 µm). **E.** Group IV. Severe necrotic, haemorrhagic areas in the liver tissue and lymphocytic cell infiltration around these necrotic foci and in the portal area (bar: 50 µm). **F.** Group IV. Severe necrotic, haemorrhagic areas in the liver tissue (bar: 20 µm).

DISCUSSION

Toxicological studies with BHT supplementation to animal feed found that adverse effects were dose-dependent. BHT amounts in excess of 526 mg/kg/day resulted in pleural and peritoneal haemorrhage in laboratory rats (Takahashi & Hiraga, 1978). A human consumes approximately 0.1 mg/kg/day of BHT. Research shows that 500 times (50 mg/kg/day) this amount yields no deleterious effects (Brannen, 1975).

AST, ALT and LDH are intra-cellular enzymes that play a role in amino acid and carbohydrate metabolism. These enzymes are present in high concentrations in the liver, muscle and brain. During liver damage, transport functions of hepatocytes are impaired and serum enzyme activity increases due to leakage from the plasma membrane. Elevated activities of AST, ALT and LDH in the serum indicate liver necrosis or disease (Jang *et al.*, 1999; Lin *et al.*, 2007).

We observed that serum AST, ALT and LDH activities increased in all groups following BHT administration. The increase in AST, ALT and LDH activities were statistically significant in Groups III and IV. The increase in liver enzyme activities occurred in a dose dependent manner. The present findings correlate with the reports of many studies using different doses of BHT (Nakagawa *et al.*, 1984; Nakagawa, 1987; Ip & Ko, 1996; Devi *et al.*, 2003; Farag *et al.*, 2006; El-Anany & Ali, 2013; Panicker *et al.*, 2014). On the other hand, some studies reported that none of tested BHT doses led to difference in serum transaminase activities (Jaeschke & Wendel, 1985; 1986; Jang *et al.*, 1999). Yamamoto *et al.* (1995), also did not observe any difference in AST and ALT activities in the

blood when rats were fed a diet containing 0.2 % BHT.

The liver has many defense and adaptation mechanisms against oxidants and xenobiotics. It has known that GST catalyses the conjugation of the biological nucleophile GSH with many electrophilic compounds. That is to say, GSH and GST play an important role in the detoxification of xenobiotic compound containing the carcinogen (Nakagawa *et al.*, 1981). Maintenance of mitochondrial GSH redox is of great importance in order to prevent chemical hepatic injury as the decrease in mitochondrial GSH-related protein thiols is critical for cell viability (Ip & Ko, 1996). The activated metabolite of BHT formed by the microsomal monooxygenase system is bound to the thiol compounds (Mizutani *et al.*, 1987; Witschi *et al.*, 1989; Devi *et al.*, 2003). The induction threshold of the glutathione conjugation system in liver is greater than or equal to 100 mg/kg for BHT (Jaeschke & Wendel, 1986).

It has been reported that oral administration of 500 mg/kg of BHT (Nakagawa *et al.*, 1981) and dietary intake of 5% BHT (Cha & Heine, 1982) elevated the total level of GSH and the activity of GST, but has not effect on GPx activity (Nakagawa *et al.*, 1981). It was observed that liver GSH level was decreased and did not alter GST activity after the application of BHT (Nakagawa *et al.*, 1984). Awasthi *et al.* (1983) reported that 0.4 w/w BHT feeding increased liver GST activity and did not alter GPx activity

In a study investigating the effect of adding BHT in two different rodent species, the liver GR activity increased significantly only in rats and GST activity – in both mice and rats (Jaeschke & Wendel, 1985). Also, other studies reported that the addition of 4000 and 1000

ppm BHT significantly enhanced GST activity of turkeys' liver (Klein *et al.*, 2002; Coulombe *et al.*, 2005). It was reported that 3 mmol/kg BHT application decreased liver GSH level and increased GR activity (Ip & Ko, 1996). The liver GPx and GST activities were increased after dietary supplementation of 0.05% and 0.5% BHT (Jang *et al.*, 1999). The liver GPx activity decreased following application of BHT at 1000 mg/kg dosage (Lin *et al.*, 2007).

In the our study, twenty eight days after the administration of 250 mg/kg of BHT, no noteworthy change was observed in GSH level and the GPx, GST and GR activities in rat liver. The lack of agreement among various studies including our results with respect to antioxidant parameters may be likely due to differences in dosage, age, periods of experiment, even animal strain and gender (Egaas *et al.*, 1995). However, twenty eight or four days after the administration of 500, 1000 and 1500 mg/kg of BHT the GSH level and GPx, GST, GR activities significantly decreased. The decreased liver activity of GPx due to BHT administration might result from the involvement of this enzyme in the dismutation of peroxides generated during BHT biotransformation. Because GSH is utilised in the GPx-mediated reactions, an enhanced intensity of these reactions might also be a cause of decrease in GSH concentration in the liver of the BHT treated rats. In a reaction catalysed by GPx, an oxidised form of GSH: GSSG is formed. Under the influence of GR and reduced nicotinamide adenine dinucleotide phosphate (NADPH), GSSG is reduced to GSH (Moniuszko-Jakoniuk *et al.*, 2007). The decrease in liver GR activity in rats treated with BHT might result from this enzyme utilisation for GSSG reduction or from NADPH

deficiency. GST catalyses reactions of toxic substances conjugation with GSH (Jurczuk *et al.*, 2006). The decreased GST activity in the liver of rats exposed to BHT alone suggests that the reduction of GSH concentration in this organ might result from GSH utilisation in antioxidative reactions. These liver biochemical alterations were accompanied by amelioration of histopathological degenerative changes seen in liver of BHT administered rats.

Several studies indicated that BHT caused liver damage (Nakagawa *et al.*, 1981; Lanigan & Yamarik, 2002). The hydropic degeneration (Klein *et al.*, 2003), vacuolar degeneration and hepatocellular degeneration including fuzzy swelling (El-Anany & Ali, 2013) and necrosis (Powell *et al.*, 1986) were reported. Contrary to the above mentioned studies, Jang *et al.*, (1999) and Lin *et al.*, (2007) showed that BHT caused no histopathological changes in liver. In this study, the livers of rats in the Group I had normal appearance. The histopathological degenerative changes in liver tissue were observed as congestion, degeneration, necrosis, haemorrhages in the other BHT-treated groups. The severity of these changes increased as a function of applied dose of BHT.

As a result, it can be said that BHT plays a role in producing liver damage in rats with depressed hepatic antioxidants. The hepatotoxic response seemed to be dose- and time- dependent.

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