

Hepatotoxicity induced by the anti-oxidant food additive, butylated hydroxytoluene (BHT), in rats: An electron microscopical study

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Summary. The anti-oxidant food additive, butylated hydroxytoluene (BHT), was fed to Sprague-Dawley rats at three concentrations: 0.2%, 0.4% and 0.8% for periods of 6, 12, 18 and 24 weeks, and the results were compared with corresponding groups treated with a potent carcinogen, 7,12-dimethylbenz[a]anthracene (DMBA) groups, with olive oil, and with untreated control groups. BHT resulted in a significant increase in liver weight. The liver cells presented gradual vacuolization, cytoplasmic disintegration, "moth-eaten" appearance, ballooning degeneration, hepatocellular necrosis, aggregation of chromatin material around the periphery of the nuclear envelope, SER proliferation, RER clumping with broken cisternae, withered and autolyzed mitochondria, augmentation of lipid droplets and glycogen depletion. On the other hand, there was no sign of tumorigenicity. Whether or not BHT acts as a carcinogen in long-term administration may depend not only upon the organ system examined, but also on the strain of the animal used.

Key words: BHT, Hepatocyte, Necrosis, DMBA, Rat, Electron Microscopy

Introduction

Since 1954, butylated hydroxytoluene has been one of the most widely used preservatives added to food, acting as anti-oxidant in foods containing fats and oils and in food packaging (Concon, 1988). Butylated hydroxytoluene (BHT), also termed 2,6-di-*tert*-butyl-*p*-cresol or 2,6-di-*tert*-butyl-4-methylphenol, has a molecular weight of 220.34, with a chemical formula of C₁₅H₂₄O (Babich, 1982). The National Research Council (1972) of the US National Academy of Sciences as well as the Bureau of Foods (1973) reported that BHT was used in jams, jellies, sweet sauces, nut products,

dairy products, meat products, snack foods, hard and soft sweets, gelatin pudding, baked foods, frozen dairy products, alcoholic and non-alcoholic beverages, soups, processed vegetables and fruits and edible fats and oils. The daily intake of BHT has been set by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 1967) at a maximum of 140 mg/day for a 70 kg man which is equivalent to 0.01%. In the United States and the United Kingdom the daily intake averages about 2 and 1 mg/person/day, respectively (Karma, 1973). JECFA (1986), have recently lowered the acceptable daily intake (ADI) for BHT from 0.00-0.50 to 0.00-0.125 mg/kg body weight. Many European countries, Canada, United States, South Africa and Kuwait are some of the countries that permit BHT in foods.

In rats, large doses of BHT (700-1000 mg/kg body wt.) produce hepatic centrilobular necrosis, increased serum transaminase activities and hemorrhagic death (Nakagawa et al., 1984). Substantial evidence links hepatotoxicity to BHT metabolism, with toxicity varying with species (e.g. rats respond, while mice and hamsters do not) (Takahashi et al., 1980). Though generally considered to be safe at the acceptable daily intake (ADI), the liver, lungs and blood are the main targets of BHT toxicity. Ultrastructural studies carried out by Crampton et al. (1977) have shown centrilobular enlargement of hepatocytes in rats given 0.4% BHT for 18 months. Proliferation of the smooth endoplasmic reticulum (SER) within the centrilobular hepatocytes was evident after 1 week of treatment. There was little further change in the appearance of the SER.

Hemorrhagic death with a decrease in the prothrombin index occurred in Sprague-Dawley, Wistar, Dorryu, and Fischer strains of rats given 1.2% BHT in their diets (Takahashi et al., 1980). Hirose et al. (1981), used BHT at a concentration of 0.25% or 1% for 104 weeks. This dose brought about a significant reduction in body weight-gain, as well as relative spleen weight and white blood cell count. BHT-treated animals of both sexes also showed increased relative liver weight and total blood cholesterol. Furthermore, tumours were found in the liver, pancreas, mammary glands, uterus,

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pituitary gland and adrenal glands. Sprague-Dawley rats treated with 250 mg/kg body wt BHT, showed an increase in the size of liver cell nuclei (Romagna and Zbinden, 1981). At 1.20% level, for a period of one week, BHT caused a significant decrease in the hepatic and plasma triglycerides and a significant increase in the hepatic phospholipids (Takahashi and Hiraga, 1981). When 0.5% BHT was used, it had caused retardation of growth which, according to Thamavit et al. (1985), was due to induction of enzymes participating in anti-oxidant metabolism. Higher doses of BHT (500 mg/kg body wt) given by gavage for 28 days to rats, caused periportal hepatocyte necrosis, proliferation of bile ducts, fibrous and inflammatory cell reactions (Powell et al., 1986). Olsen et al. (1986), Wurtzen and Olsen (1986) and Inai et al. (1988), have shown an increased incidence of liver tumours in BHT-treated Wistar rats and B6C3F1 mice. At a level of 0.2%, BHT caused liver enlargement by 24%, elevation in cholesterol, and increased hepatic microsomal lipid peroxidation (Yamamoto et al., 1995). It was shown by Thompson and Moldeus (1988), that BHT effectively dissipated membrane potential across the mitochondrial membrane and caused the release of calcium ions and mitochondrial swelling. These mitochondrial deformities were reflected by a rapid decrease in ATP levels in intact hepatocytes which preceded cell death. It also causes inhibition of inter-cellular molecular exchange between cultured liver cells (Williams et al., 1990), indicating that BHT has an epigenetic effect on cell membrane function. BHT fed to male mice over a period of 7 days caused congestion of capillaries and small blood vessels, increased cellularity and diffuse thickening of alveolar septa (Waseem and Kaw, 1994). It was reported to induce a significant increase of chromatid and chromosome breaks. In anaphase-telophase, it has been observed to induce a significant increase of multipolar mitosis (Grillo and Dulout, 1995).

The toxicology of the anti-oxidant BHT has become

one of the more controversial areas in the continuing debate on the safety of food additives. Thus, it appears to be an interesting model substance for experimentation with which more light could be shed on the ultra-structural changes in the liver of Sprague-Dawley rats. In the present study, experiments were designed at several time intervals: 6, 12, 18 and 24 weeks of BHT feeding and the results were compared with those obtained after the administration of 7,12-dimethylbenz[a]anthracene (DMBA) as a potent carcinogen, or olive oil as well as untreated control groups.

Materials and methods

Animals and housing conditions

Specified pathogen-free male Sprague-Dawley rats (6-10 wk old, 91-173 g) were obtained from the breeding unit of the Department of Biological Sciences, University of Kuwait. They were housed, four per stainless steel wire cages (24x41x19 cm), with sterilized softwood chips as bedding. Housing conditions were maintained at a temperature of 21±1 °C, with alternate 12 hour light/dark cycles.

Experimental protocol and diet preparation

The actual dose level of BHT in the prepared diets was chosen on the basis of previous literature e.g. Meyer and Hansen (1980), and on the short-term prehepatotoxicity tests (LD₅₀ evaluation study). Also, a decision-point approach to hepatotoxicity tests was performed on a list of food additives including Tartrazine (E102), Amaranth (E123), Erythrosine (E127), Allura red (E129), Indigo carmine (E132), Sodium nitrite (E250) and Potassium bromate (E924) (personal communication).

A total of 60 rats were assigned randomly to six groups, each group consisting of 10 rats. The test

Table 1. Treatment schedule of 6-10 week old rats, showing the total and weekly Chemical Intakes of various doses of chemicals in diet for 24 weeks.

GROUP	TEST CHEMICAL	DOSE LEVEL (%)	DOSE LEVEL (mg/kg of diet)	WEEK			
				6wk	12wk	18wk	24wk
Chemical intake (total mg/kg body wt)							
I	BHT	0.2	1999.92				
II	BHT	0.4	3999.75	12559.20	23025.20	28258.20	33491.20
III	BHT	0.8	8000				
IV	DMBA	0.05	500	66.66	133.33	199.99	266.66
V	Olive oil	3.00	30.57 ^a	15	30	45	60 ^b
Chemical intake (weekly mg/Kg body wt)							
I	BHT	0.2	1999.92				
II	BHT	0.4	3999.75	2093.20	1918.77	1569.90	1395.47
III	BHT	0.8	8000				
IV	DMBA	0.05	500	11.11	11.11	11.11	77.77
V	Olive oil	3.00	30.57 ^a	2.5	2.5	2.5	2.5 ^b

^a: dose level expressed as ml/kg of diet; ^b: chemical intake expressed as ml/kg body wt.

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chemicals butylated hydroxytoluene (BHT) (ATC Medical Technics, Germany), 7,12-dimethylbenz[a]anthracene (DMBA) (Sigma, USA) and olive oil were stored in sealed containers. The basal diet (SDS rodent pellets, England) was stored in an environmentally-controlled room. Diets were prepared and presented weekly to rats as follows:

group I	-	received 0.2% BHT
group II	-	received 0.4% BHT
group III	-	received 0.8% BHT
group IV	-	received 0.05% DMBA
group V	-	received 3.00% Olive oil
group VI	-	controls

Table 1 summarizes the treatment schedule of rats, 6-10 week old, treated for 24 weeks with various doses of test chemicals mentioned previously. In groups I, II and III, BHT was dissolved in olive oil (0.5233 gm/1.5 ml olive oil/rat/week) and was incorporated into the food pellets at concentrations of 0.2%, 0.4% and 0.8% respectively (total dose intake at each dose level: 33491.2 mg/kg body wt, 24 weeks). In group IV, DMBA was dissolved in olive oil (1.66 mg/0.166 ml olive oil/rat/week) and then stored at 0-4 °C for at least one hour. The mixture was then incorporated into the food pellets at concentration of 0.05% (total dose intake: 266.666 mg/kg body wt, 24 week) by placing 1-2 drops of it on each pellet. Experimental animals in group V were treated with 3.00% of olive oil in the basal diet (total dose intake: 60 ml/kg body wt. or 36 ml/rat, 24 week). Control animals were fed with SDS standard rodent food (England) and had water *ad libitum*. The SDS standard mixture contained 4.98% fat, 21.25% protein, 4.75% fibre, 6.25% moisture, 4.4% ash, 10% sugar, and 48.33% starch. The treated animals were also given the same diet with addition of appropriate test chemicals. Normal basal diet was given until the termination of experiment (week 24). Body weights were measured at 2-weekly intervals. At the following time points: 6, 12, 18, 24 weeks, 2 rats for each group were subjected to a single intraperitoneal injection with a 25% urethane (0.6 ml/100 gm body wt.). After anaesthesia with urethane, the abdomen was opened and lobes of the liver were quickly removed, weighed and fixed in cold 3% glutaraldehyde, and routinely processed for electron microscopy examination.

Electron microscopy studies

Preparation of ultra-thin sections for electron microscopy

Soon after rapidly excising the liver, cold 3% glutaraldehyde (BDH, England) in 0.2M cacodylate buffer with 0.2M sucrose (pH 7.2) was dropped over its surface. The liver was then chopped into small cubes (1 mm) using a sharp blade, and immediately placed in a vial containing the same fixative overnight, at 0-4 °C on a rotator. The specimens were washed with 0.2M sodium

cacodylate buffer at pH 7.4 (TAAB, England) and kept overnight, with two renewal changes, then post-fixed in 1% osmium tetroxide (Fluka, Germany) (in 0.2M cacodylate buffer pH 7.4) for one hour at room temperature. The specimens were washed twice with the same buffer for 20 minutes, and dehydrated in a graded series of ethanol, then two changes of absolute alcohol, for 15 minutes each. They were then transferred through two changes of propylene oxide (TAAB, England), 5 minutes each, to propylene oxide:Araldite M (Fluka, Germany) (1:1), for 1 hour and in pure Araldite M overnight at room temperature, then embedded in pure Araldite M for 48 hours at 60 °C.

One micrometer thick sections were cut, stained with toluidine blue and examined under the light microscope. Ultrathin sections were cut and mounted on 300 mesh copper grids and stained with uranyl acetate followed by lead citrate. The sections were examined in a JEM-1200 EXII Jeol electron microscope (Japan) operated at 60-80 kV. Micrographs were taken on Kodak electron microscopy film, NY Estar with a dimension of 8.3x10.2 cm. Prints were made on Kodak Polymax glossy paper.

Statistical analysis

A computer-assisted assessment was made for the differences between control and treated animals with BHT and DMBA with respect to the mean body weights and organ weights using the independent t-test. Quantitative analysis of hepatic damage in all groups was performed by measuring four basic cellular parameters: cell size, nuclear size, number of cells with damaged cytoplasm and the number of cells with damaged nuclei. Five hexagonal lobules were chosen randomly for each liver section. From different areas of each lobule, the cell and nuclear size of 10 randomly-selected cells were measured, using an eye piece graticule at 40x magnification. A total of 50 readings were taken and expressed as means with standard deviations (\pm SD). Statistical analysis was done by independent t-test and analysis of variances using the SPSS plus software program. The 0.05 and 0.01 levels probability were the criteria of significance.

Results

Effect of BHT on body and liver weights

The body weights of the animals after treatment with three different dose levels of BHT did not vary significantly from those of the control groups (Table 2).

On week 6 of the experiment, the organ weights of rats subjected to 0.2%, 0.4% and 0.8% BHT, were significantly different from those of the controls ($p < 0.05$). There was also a significant gain in organ weight in week 18 (0.8% BHT). On the other hand, no differences were found in week 24. Table 2 also depicts the relative liver weight gain in the three concentrations of BHT tested.

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Table 2. Experimental design of liver treatments and effects of three different concentrations of BHT on animal and organ weights.

GROUP TREATMENT (n=10)	DOSE LEVEL %	BEFORE TREATMENT bw (g)	AFTER TREATMENT bw (g)				RELATIVE LIVER WEIGHT (g/100g)	
			6 wk	12 wk	18 wk	24 wk	Initial	Gain
Control	0	143±7	336±8 ^a	450±19	516±17	552±32	2.4±0.8	3.3±1
			8.2±0.8 ^b	15±5	21±0.9	18±0.5		
Olive oil	3	136±10	377±8 ^a	509±25	531±2	560±21	3.4±6	3.5±2
			13.5±0.5 ^b	19±2	18±0.5	20±0.5		
Experimental	0.2	112±12	360±32 ^a	447±38	6213±33	608±34	2.6±1.8	3.4±.8
			16.6±0.6 ^{**a}	17±0.6	18±1.5	20±0.7		
			16±.6 ^{**b}	19±0.8	29±1	23±1		
	0.4	102±8	360±31 ^a	426±13	645±14	593±25	2.4±1.8	4±.4
			16±.6 ^{**b}	19±0.8	29±1	23±1		
			21±0.5 ^{**b}	24±0.7	27±0.7 ^{**}	26±2.5		
DMBA	0.8	107±7	349±24 ^a	442±21	547±14	528±34	6.5±.2	5±.4
			21±0.5 ^{**b}	24±0.7	27±0.7 ^{**}	26±2.5		
			21±0.5 ^{**b}	24±0.7	27±0.7 ^{**}	26±2.5		
DMBA	0.05	118±5	307±15 ^a	431±6.6	436±52	373±27	3.2±.6	3.2±.3
			10±0.9 ^a	15±.1.9	17±0.1	12±1 ^{**}		

Values are expressed as means±SD and are significantly different from control at 0.05 level independent t-test**. n: number of animals per group. Dose level: the given dose expressed as a percentage (0.2%, 0.4% and 0.8%) for 24 hours. bw (g): body weight of animals in grams per group before treatment. bw (g) body weight of animals in grams at the onset of the dosages. ^a: body weight of animals in grams. ^b: liver weight (g) No mortality through the experiment.

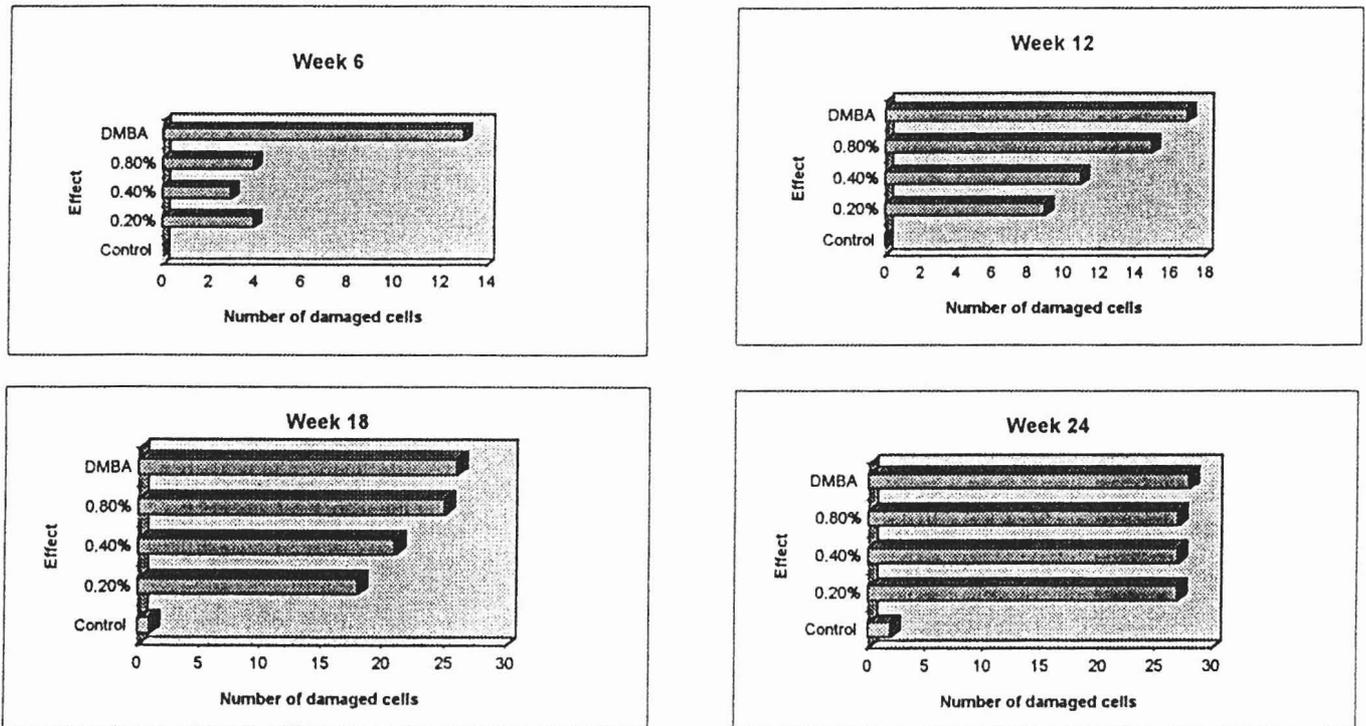


Fig. 1. Cellular damage in BHT concentrations and DMBA-treated liver cells.

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Effects of BHT on cell and nuclear sizes

Butylated hydroxytoluene administered to rats for 6 weeks at 0.2% level, resulted in a significant decrease ($p<0.01$) in cell size, when compared with the respective controls. However, hepatocellular hypertrophy was

evident, with the greatest differences ($p<0.01$) observed on week 12 (0.4% BHT), week 18 (0.2% and 0.4% BHT) and week 24 (0.2% and 0.8% BHT). There was, however, no significant difference between the nuclear sizes, within the three concentrations of BHT (Table 3). It is clear from the above results that increased BHT

Table 3. Measurements of cell and nuclear sizes in liver sections after treatments with BHT at three different concentrations.

GROUP TREATMENT (n=50)	CELL SIZE (μm ; mean \pm SD)				NUCLEAR SIZE (μm ; mean \pm SD)			
	Number of cells with damaged cytoplasm				Number of cells with damaged nuclei			
	6 wk	12	18 wk	24 wk	6 wk	12 wk	18 wk	24 wk
Control	17 \pm 1.7 0	16.6 \pm 2 0	17.5 \pm 1.8 1	19.7 \pm 1.5 2	6.7 \pm 1 2	7.7 \pm 1 6	7.8 \pm 9 7	7.7 \pm 0.1 9
Olive Oil	17.2 \pm 1.6 0	20.4 \pm 2.5 2	23.3 \pm 2.5** 4	23.3 \pm 3.7** 4	7 \pm 1 3	6.8 \pm 1.5 5	8.2 \pm 9 12	7.8 \pm 8 14
BHT 0.20%	15.3 \pm 1.5** 4	18 \pm 3 9	20.7 \pm 2** 18	22 \pm 9** 21	6.8 \pm 1 10	7.7 \pm 9 12	7.7 \pm 1 15	7.8 \pm 9 20
BHT 0.40%	17 \pm 2 3	19.4 \pm 2.7** 11	21.7 \pm 3.4** 21	22.2 \pm 5.2* 27	6.5 \pm 8 13	8 \pm 9 15	8.9 \pm 1 18	7.8 \pm 7 21
BHT 0.80%	15.7 \pm 1.7 4	20.8 \pm 2.5** 15	24.5 \pm 3.4** 25	23.5 \pm 3** 27	6.7 \pm 1 12	7.6 \pm 1 16	7.9 \pm 8 19	8 \pm 1 21
DMBA	17.7 \pm 2 13	17.7 \pm 3 17	23.2 \pm 3.5** 26	25.5 \pm 3.5** 28	7 \pm 6 14	7 \pm 6 17	7 \pm 6 21	7 \pm 9 20

Data were evaluated by independent t-test at significance level 0.05*, (**) is highly significant at 0.01 level. n: number of readings per group

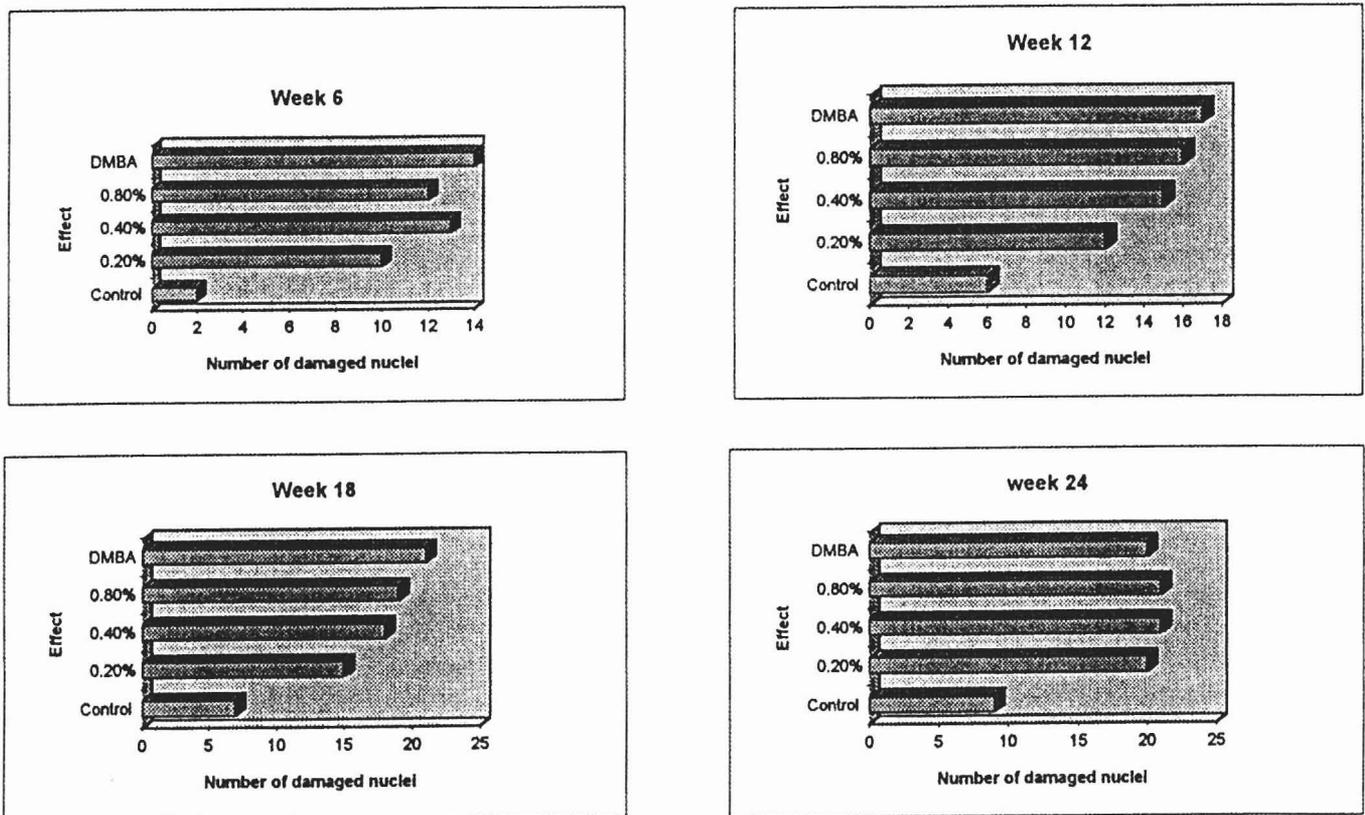


Fig. 2. Nuclear damage in BHT concentrations and DMBA-treated liver cells.

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Table 4. Summary of characteristics of cell lesions after 6-24 weeks of treatment of rats with BHT against DMBA.

GROUP TREATMENT (n=50)	SPECIFIC ALTERATIONS IN CELL ORGANELLES, LIPID AND GLYCOGEN INCLUSIONS						
	Mitochondrial aberrations	Nuclear disintegrations	Cytoplasmic vacuolizations	SER proliferations	RER fragmentations	Lipids augmentations	Glycogen depletions
BHT	+++	++	++	++	++	+	+++
DMBA	++	++	+++	+++	+++	+++	+++

(+) and (-) indicate relative presence or absence of respective feature. Observations which cover 0.2%, 0.4%, and 0.8% BHT concentrations are statistically non significant. DMBA concentration was 0.05%.

concentrations and treatment durations had a gradual but damaging effects on the cells as well as their nuclei as compared to controls. The greater effects of DMBA may be taken as an indication of toxicity (Figs. 1, 2).

Electron microscopy

0.2% BHT

On the 6th week, aggregates of chromatin around the periphery of nuclei were noticed. The cytoplasm of some cells exhibited a "moth-eaten" appearance due to the disruption of cell organelles. In some areas, the rough endoplasmic reticulum (RER) appeared dense (Fig. 3). By week 12, some hepatocytes were mostly anucleated, while others exhibited degenerated nuclei. Mitochondrial diffusion and autolysis were evident. A few mitochondria of dumbbell-like and other shapes were also noted (Fig. 4). After 18 weeks of treatment, some hepatocytes were anucleated or with degenerated nuclei. Some areas in the cytoplasm showed a prominent smooth endoplasmic reticulum (SER) proliferation with moderate "moth-eaten" appearance (Fig. 5). By week 24, continuation of cytoplasmic disruption with a striking proliferation of SER was apparent. The RER had become clumped. Some segments of RER were in close association with or encircled the mitochondria. Lipid droplets were augmented and glycogen particles were depleted (Fig. 6).

0.4% BHT

The hepatocytes of BHT-exposed animals in this

group, showed ultrastructural alterations to a greater extent than the previously-mentioned group (0.2%). By the 6th week, the dense stacks of the RER were scattered. Some of them were in a close association or encircled the mitochondria. Some mitochondria were dumbbell-shaped (Fig. 7). By week 12, cytoplasmic and nuclear degeneration which first started in 0.2% BHT-treated animals (12 week) were also evident. Much of the RER were clearly seen to have broken cisternae. The mitochondria were in an early stage of developing a diffuse structure (Fig. 8). After 18 weeks of treatment, clumping of chromatin was clearly observed. Large zones of proliferated SER were seen (Fig. 9). By week 24, the hepatocyte image revealed maximal cytoplasmic disintegration with lipid droplet augmentation and glycogen depletion. Deteriorated mitochondria were prominent (Fig. 10). This phenomenon was highly comparable to what was observed in 0.2% BHT-treated animals (12 week), presented in Fig.4. The characteristics of cell lesions from weeks 6 to 24 of BHT administration, in comparison to the carcinogen DMBA, are depicted in Table 4.

0.8% BHT

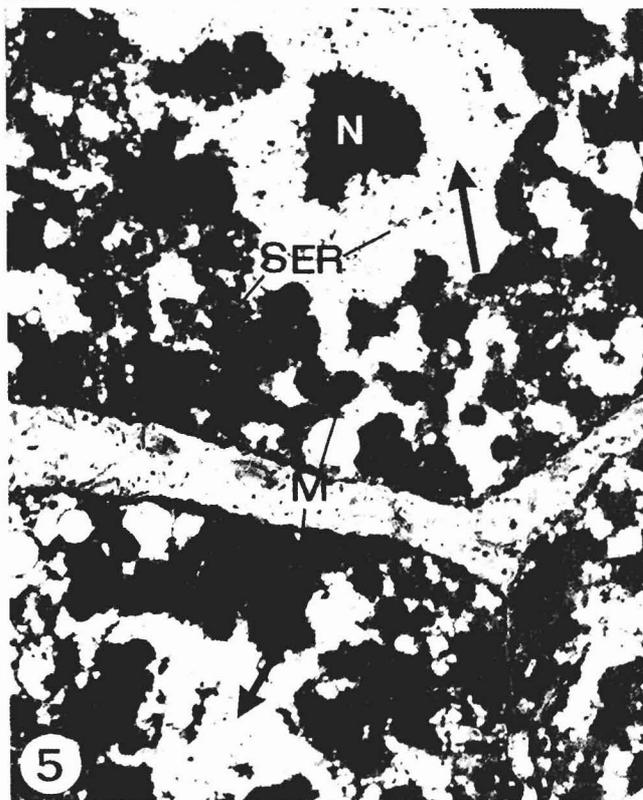
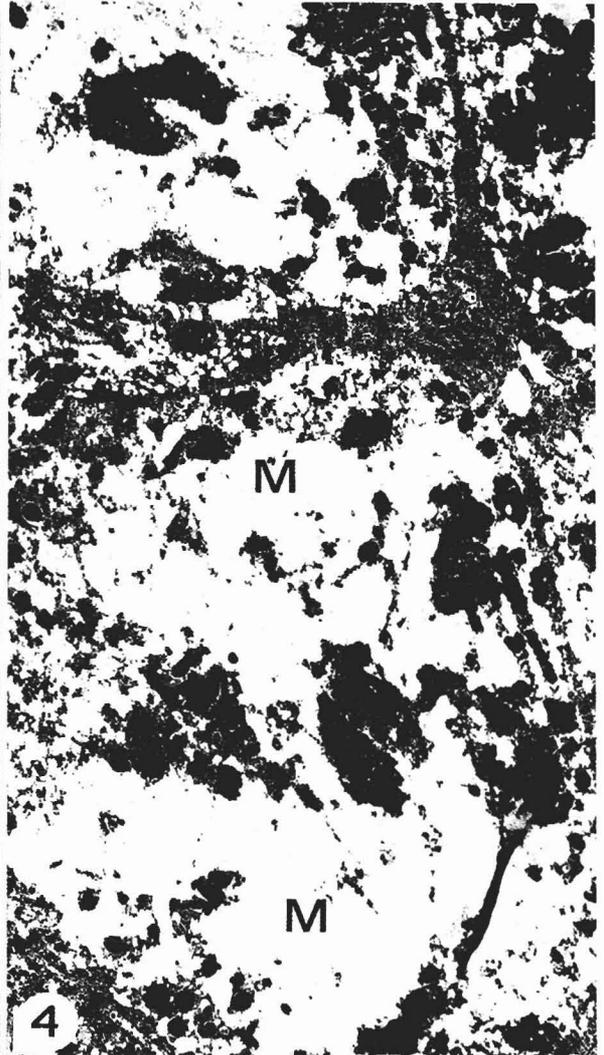
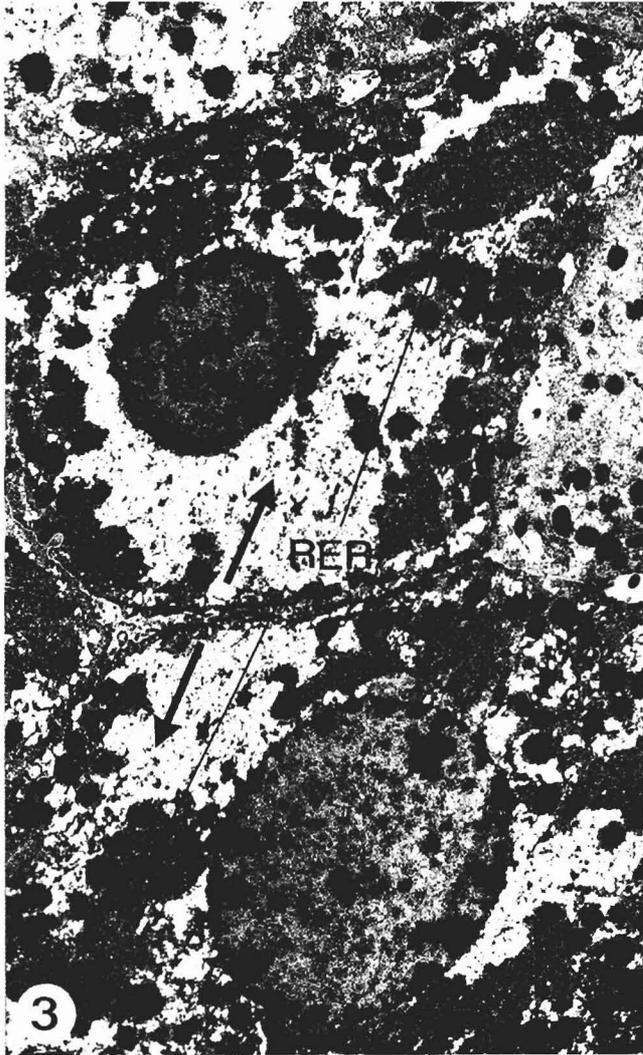
In contrast to the observations made in the previous group, ultrastructural examination of liver specimens from a number of BHT-treated animals (0.8%) demonstrated a wide range of effects, from moderate to severe alterations. By the 6th week, moderate changes were observed in the cytoplasm of some hepatocytes, characterized by the "moth-eaten" appearance. Moderate

Fig. 3. Electron micrograph of a portion of two hepatocytes from rat treated with 0.2% BHT for 6 weeks. Note the build up of chromatin at the periphery of the nucleus (N). A "moth-eaten" appearance of the cytoplasm is observed (arrows). In some areas there are dense profiles of rough endoplasmic reticulum (RER). UA/Pb stain. x 8,000

Fig. 4. Electron micrograph of a portion of liver from rat treated with 0.2% BHT for 12 weeks. Some hepatocytes are anucleated. Many mitochondria (M) are diffuse in appearance and autolysed. UA/Pb stain. x 6,000

Fig. 5. Electron micrograph of a portion of hepatocytes from rat treated with 0.2% BHT for 18 weeks. Some hepatocytes are anucleated or with delimited nuclei (N). The cytoplasm depicts prominent proliferation of smooth endoplasmic reticulum (SER), with moderate "moth-eaten" appearance (arrows). Alterations in the hepatocyte mitochondrial diffusion (M) are observed. UA/Pb stain. x 8,000

Fig. 6. Electron micrograph of a portion of hepatocyte from rat treated with 0.2% BHT for 24 weeks. Moderate cytoplasmic vacuolization is evident (arrows). The picture shows clumping of rough endoplasmic reticulum (RER) which are seen close to or encircling some of the mitochondria (M). Some lipid droplets (LD) are augmented. UA/Pb stain. x 10,000



increases were observed in the number of SER. Many mitochondria appeared abnormal and were transformed primarily from the normal state to a withered form (Fig. 11). By week 12, severe cytoplasmic vacuolization, "moth-eaten" appearance was evident. The SER was highly proliferated, while the RER was often distended and tend to encircle the mitochondria (Fig. 12). After 18 weeks of treatment, many cytoplasmic areas were degenerated. Mitochondria were aggregated; their morphology was similar to that described previously (0.4% BHT, 24 weeks) (Fig. 13). On week 24, the chromatin of the nuclei was clumped. Advanced stage of cytoplasmic vacuolization and SER proliferation were observed. Lipid droplets were present, while glycogen particles were completely depleted (Fig. 14).

0.05% DMBA

Figure 15 depicts an electron micrograph from a rat treated with 0.05% DMBA for 12 weeks. Nuclear damage was evident. The hepatocyte cytoplasm was severely disintegrated. The SER was much more extensive than usual. RER was densely stacked and disrupted, and some of RER elements encircled or partially surrounded the mitochondria. Lipid droplets were significantly increased and there was no sign of glycogen particles was evident.

3.00% Olive oil

The structure of the hepatocyte from animals in this group was similar to that described below for control animals. The nucleus, SER, RER and mitochondria were normal. Lipid droplets of varying sizes were abundant (Fig. 16).

The hepatocyte of untreated rat (control) appeared normal. The spherical nucleus was limited by a clearly defined nuclear envelope. Other cell organelles such as the SER and the RER were arranged in parallel profiles and scattered randomly throughout the cytoplasm. The mitochondria were round to elongated with prominent cristae. Glycogen particles were normally distributed in the cytoplasm (Fig.17).

Discussion

Although most food additives (preservatives) are now considered to be without potential adverse effects, and are thus classified as Generally Recognized as Safe (GRAS), there have been problems concerning the safety of some of these chemicals. Thus, it was with this background coupled with the potential side-effects of food additives in the diet of mankind and on the cell ultrastructure, including possible rodent hepatotoxicity due to butylated hydroxytoluene (BHT) administration, that the present study was undertaken. BHT (E321) is considered to be an anti-oxidant food additive used in a large number of food materials, and further information on this compound is therefore of value.

BHT was selected in the present study after a pre-hepatotoxicity test programme carried out on nine food additives obtained from ATC Medical Technics (Hamburg, Germany). The screening of the nine food additives was performed in a separate study on a group of Sprague-Dawley rats (unpublished data). The food additives screened were: four colouring agents such as Tartrazine, Erythrosine, Allura Red, Indigo Carmine, one food preservative such as sodium nitrite, two uncertified food additives of which, one was a colouring agent, like Amaranth, and one improving agent, like Potassium bromate, together with BHT as an anti-oxidant food additive.

Almost all the literature on BHT's effects on hepatocytes have been focused mainly on the the biochemical (Hirose et al., 1981; Tajima et al., 1981; Takahashi and Hiraga, 1981; Nakagawa et al., 1983, 1984, 1994; Tajima et al., 1984; Powell et al., 1986; Kagan et al., 1990; Bolton and Thompson, 1991; Shertzer et al., 1991; Lok et al., 1995; Yamamoto et al., 1995) and histological aspects, at the light microscopy level (Hirose et al., 1981; Nakagawa et al., 1984; Olsen et al., 1986; Powell et al., 1986; Mizutani et al., 1987; Inai et al., 1988; Briggs et al., 1989; Kagan et al., 1990; Powell and Connolly, 1991). On the other hand, there have been few studies conducted at the ultrastructural level (Kerr et al., 1966; Lane and Lieber, 1967; Crampton et al., 1977).

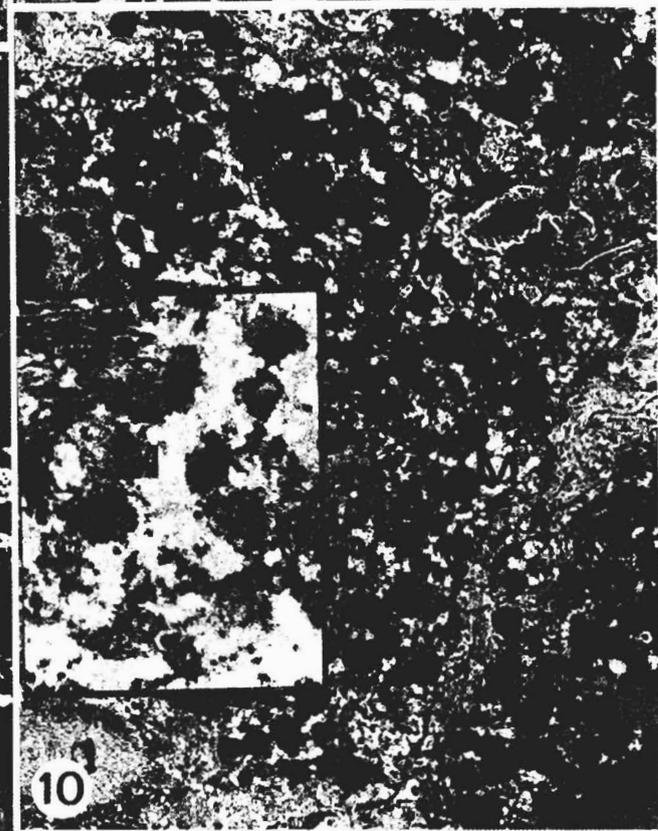
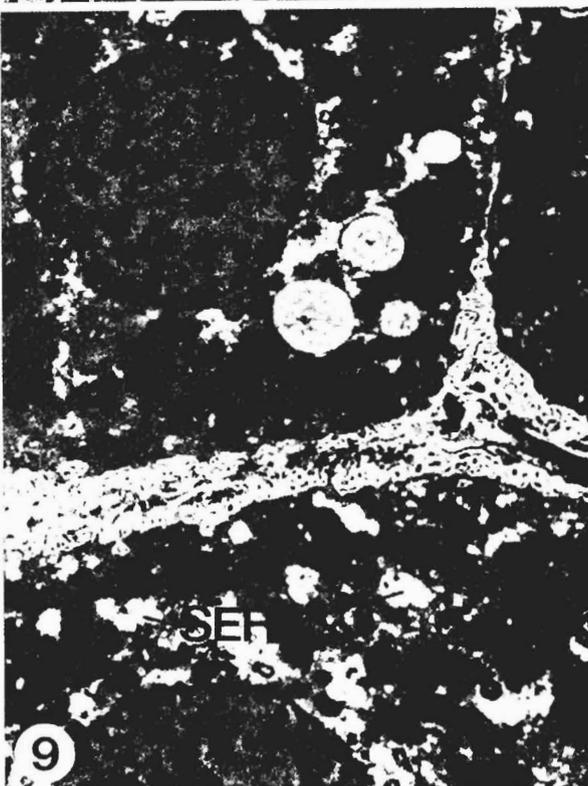
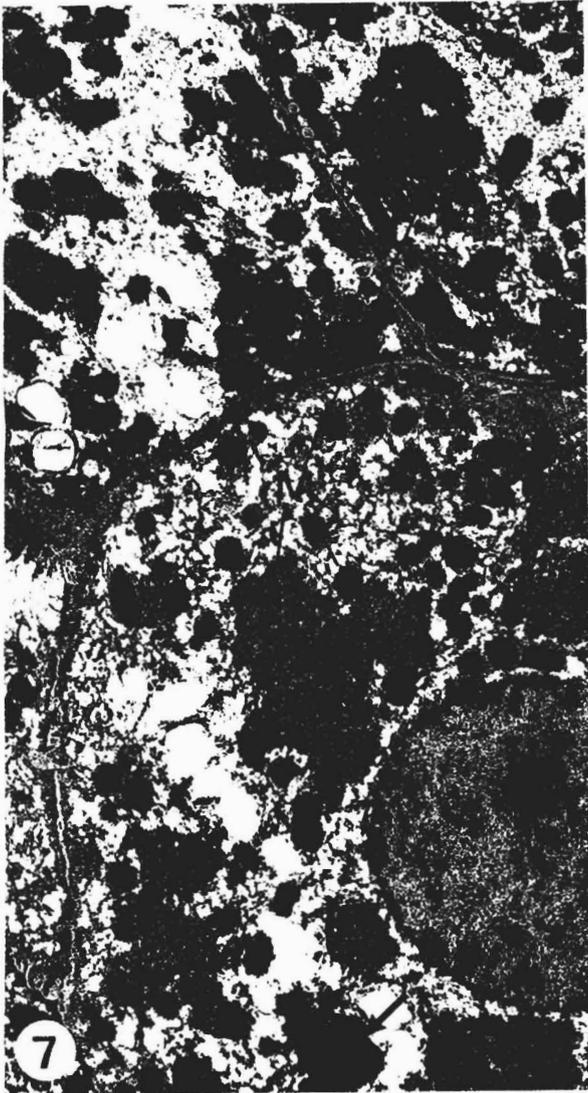
In our study, it has been shown that BHT had a

Fig. 7. Electron micrograph of a portion of hepatocyte from rat treated with 0.4% BHT for 6 weeks. The rough endoplasmic reticulum (RER) is densely stacked with long profiles woven between mitochondria (M). The mitochondria are slightly abnormal; some of them are dumbbell-shaped (short arrows). UA/Pb stain. x 8,000

Fig. 8. Electron micrograph of a portion of liver from rat treated with 0.4% BHT for 12 weeks. Note the cytoplasmic degeneration. The rough endoplasmic reticulum (RER) are disorganized with dense granules and are showing broken cisternae (Inset). The figure shows a large region containing mitochondria (M) at the onset of autolysis. UA/Pb stain. x 10,000, inset x 14,000

Fig. 9. Electron micrograph of a portion of hepatocyte from rat treated with 0.4% BHT for 18 weeks. The figure shows the nuclei (N) with clumped chromatin. The proliferation of smooth endoplasmic reticulum (SER) could be seen. UAPb stain. x 8,000

Fig. 10. Electron micrograph of a portion of liver from rat treated with 0.4% BHT for 24 weeks. The figure exhibits cytoplasmic disintegration with presence of some lipid droplets (LD). The cytoplasm is filled with proliferated smooth endoplasmic reticulum (SER). Again, many profiles of the rough endoplasmic reticulum (RER) are in close association with the mitochondria (M). The inset shows remarkably withered mitochondria. UAPb stain. x 8,000 inset x 17,500



variable effect on hepatocytes depending on the concentrations used (0.2%, 0.4% and 0.8%) as well as the duration of the dose given i.e. 6 week, 12 week, 18 week and 24 week, in comparison to the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) and control groups. Powell et al. (1986), suggested that repeated cycles of necrosis and regeneration were observed in their study on short-term administration of high doses of BHT, might produce actively replicating cells that are more vulnerable to neoplastic transformation than resting cells. In this study, the liver weight gain was observed on week 6 of the experiment in all dose-groups. There was also a significant gain in liver weights in week 18 in the highest dose-group (0.8%). Thus, hepatocellular hypertrophy was present with significant differences ($p < 0.05$) observed in week 18 (0.8%). The highly significant differences ($p < 0.01$) was observed in week 18 (0.2% and 0.4% BHT). A relationship between liver weight gain and cellular hypertrophy was observed. The increase in liver weight could be attributed to cellular hypertrophy and/or cellular proliferation (Briggs et al., 1989). The currently available data were in accordance with those of Kerr et al. (1966), Crampton et al. (1977), Powell et al. (1986) and Briggs et al. (1989). Kerr et al. (1966), Lane and Lieber (1967) and Crampton et al. (1977), suggested that the greater part of the increase in liver weight after treatment with BHT was due to increased individual cell mass consisting in large measure, of SER which tends to agree with our electron microscopy results. Khan et al. (1986), who worked on crude oil, showed that the liver weight gain is highly correlated with increased protein content. Since the RER is responsible for protein synthesis, its proliferation was served as a marker for increased total protein and RNA contents. In the present study, data from electron microscopy revealed some areas with an increase in RER profiles in the 0.2% and 0.4% BHT treatment (week 6). Interestingly, our work supports an early proposal of Pascal (1974), that BHT causes an elevation in hepatic protein and RNA contents. Ballooning degeneration and cytoplasmic disintegration were profound features of BHT-treated hepatocytes, especially when the sections were observed at a low magnification. "Moth-eaten" appearance have been a pronounced characteristic of hepatocytes, even in the early stages of

BHT feeding and at a low concentration, such as 0.2%.

Non-neoplastic lesions including hepatocellular degeneration and necrosis were noted in rats and mice exposed to BHT in their diets. The incidence of these histopathologies were dose related (Powell et al., 1986; Mizutani et al., 1987). In the present study, the presence of these non-neoplastic lesions were in agreement with the findings of Bloch et al. (1990) and Safer et al. (1997), where hepatocellular degeneration and necrosis were some of the cytopathological signs of benzene and crude oil treated rats. Nakagawa et al. (1984), reported that the toxic effects of the higher doses of BHT were ascribed to the ability of their phenoxy radicals generated by microsomal cytochrome p-450 supported reactions from their substrates (Kagan et al., 1990) to react with macromolecules and other essential targets modifying vital important cellular activities. These radical intermediates can both be suicidal for cytochrome p-450 and damaging for other neighbouring macromolecules. The observations of Yamamoto et al. (1995), suggested that free radicals formed during metabolism of BHT may result in increased hepatic microsomal lipid peroxidation, which therefore appears to be responsible for BHT cytotoxicity.

In the present study, most hepatocytes from livers treated with the highest-dose of BHT showed a maximal nuclear fragmentation by week 18. Kerr et al. (1966) and Lane and Lieber (1967), reported that BHT induces perinuclear heterochromatin aggregation. Margination of heterochromatin was also observed in some of the rats treated with BHT (Powell et al., 1986). These results are corroborated by the findings of the present study. On week 6, aggregates of chromatin around the periphery of the nucleus were eminent in hepatocytes from livers treated with 0.2%, 0.4% and 0.8% BHT. The large body of evidence of BHT genotoxicity arises from a study by Grillo and Dulout (1995), whom observed a significant increase in chromatid and chromosome breaks in Chinese hamster ovary cells.

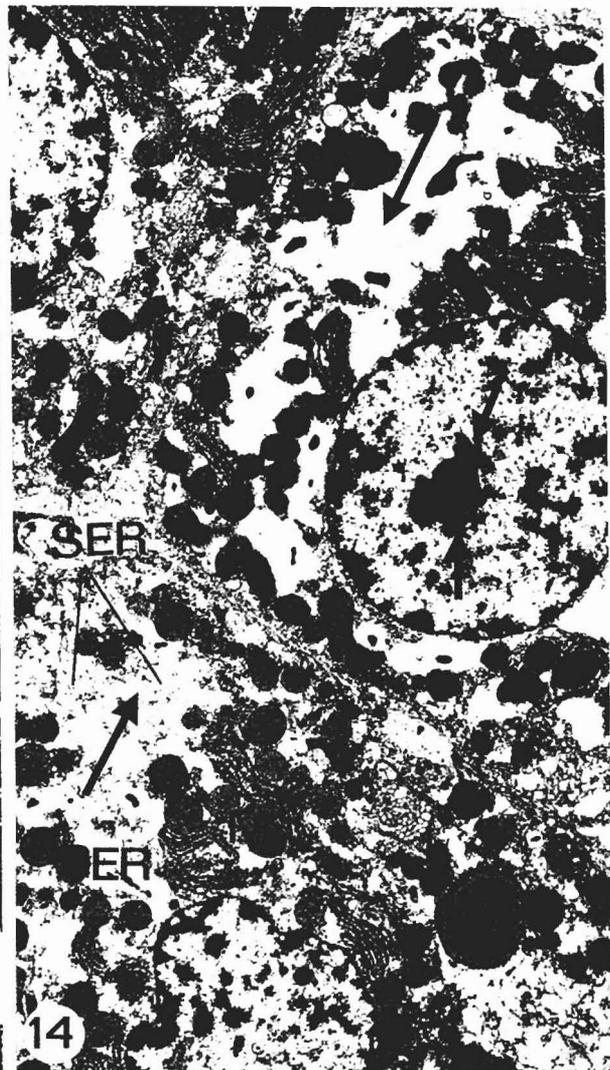
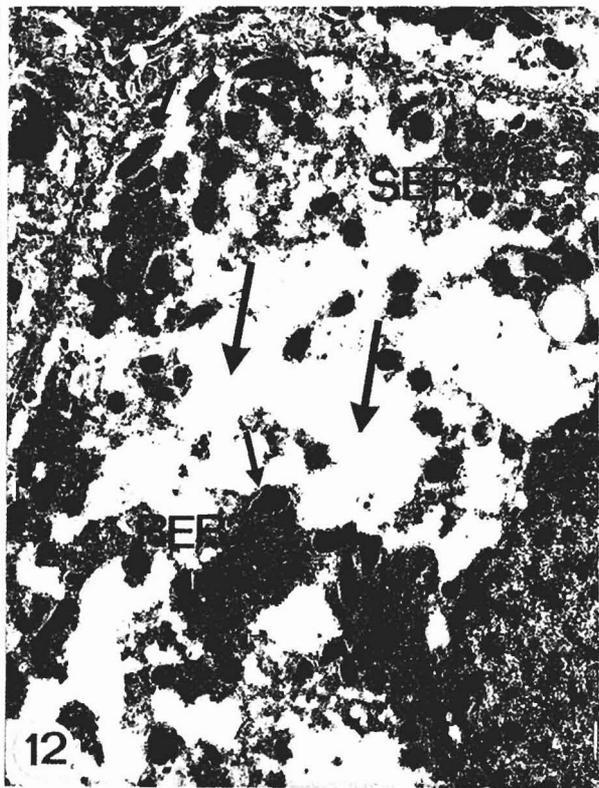
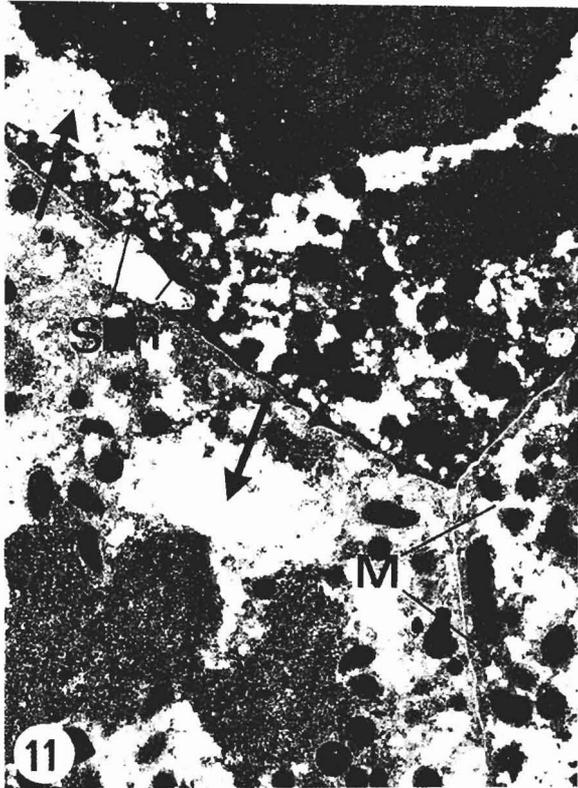
Proliferation of SER in most hepatocytes has been noted, and was consistent with the alterations reported by earlier studies on BHT (Kerr et al., 1966; Lane and Lieber, 1967; Crampton et al., 1977), and very similar to rats treated with crude oil (Safer et al., 1997), indicating that the administration of BHT induces the formation of

Fig. 11. Electron micrograph of a portion of liver from rat treated with 0.8% BHT for 6 weeks. Vacuolization of the cytoplasm is evident (arrows). The hepatocyte image reveals proliferation of smooth endoplasmic reticulum (SER). The beginning of mitochondrial transformation (M) from the normal state to the deteriorated form. UA/Pb stain. x 10,000

Fig. 12. Electron micrograph of a portion of liver from rat treated with 0.8% BHT for 12 weeks. Severe cytoplasmic vacuolization is evident (long arrows). The cytoplasm depicts more proliferation of smooth endoplasmic reticulum (SER). The rough endoplasmic reticulum (RER) cisternae, are in close contact with the mitochondria with some encircling them (short arrows). UA/Pb stain. x 10,000

Fig. 13. Electron micrograph of a portion of liver from rat treated with 0.8% BHT for 18 weeks, showing a prominent "moth-eaten" appearance of the cytoplasm (arrows). The rough endoplasmic reticulum (RER) is intimately associated with the mitochondria (M). UA/Pb stain. x 8,000

Fig. 14. Electron micrograph of two hepatocytes treated with 0.8% BHT for 24 weeks. The cytoplasm is highly vacuolized (arrows), while the chromatin is clumped (short arrows). The smooth endoplasmic reticulum (SER) is highly proliferated. The rough endoplasmic reticulum (RER) is stacked and surrounds some mitochondria (M). Lipid droplets (LD) are present. UA/Pb stain. x 8,000



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membrane system rich in the appropriate detoxifying enzymes.

In the present study a striking proliferation of SER in week 18 (0.2% BHT), and further proliferation at the high dose level (0.4%), which was directly proportional to the duration of the treatment was noticed. Also, more proliferation of SER was observed in 0.8% BHT-treated animals, which was well seen in weeks 12 and 24. This proliferation was also in accord with the observations of other authors demonstrating the proliferation of SER following administration of the hepatotoxins dinitro-O-cresol (Braunbeck and Volkl, 1991), pentachlorobiphenyl congeners n.126 or 118 (Maclellan et al., 1994a), and 3,3,4,4'-tetrachlorobiphenyl (Maclellan et al., 1994b). In a view of the association of cell enlargement with induction of drug-metabolizing enzymes and proliferation of the SER as previously mentioned, this has been regarded as a reflection of the increased functional capacity of the liver (Crampton et al., 1977). BHT-treated animals showed a depression of glucose-6-phosphatase activity (Powell et al., 1986), which Feuer et al. (1965) attributed it to disruption of the SER. Again, Crampton et al. (1977), have linked this pathway to BHT-induced hepatotoxicity. Rossing et al. (1985), reported that BHT elicited a marked extra production of H₂O₂ in the endoplasmic reticulum, which subsequently increase the formation of -OH radicals. These by-products of oxygen metabolism are currently assumed to be connected to a variety of pathological processes including lipid peroxidation, organ injury, DNA damage and neoplastic transformation (Weinke et al., 1987). By week 12 (0.4% BHT), the RER showed broken cisternae, which were in accord with Safer et al. (1997).

A diffused appearance and autolysis of mitochondria was pronounced in week 12 (0.2% BHT). In week 12 (0.4% BHT), the onset of transformation of mitochondria from normal state to withered form was evident. In week 24 (0.4% BHT), on the other hand, remarkably autolyzed mitochondria were clearly seen. This was in harmony with observations reported in rats administered with crude oil (Safer et al., 1997). The different mitochondrial shapes exhibited structural abnormalities that may affect their function, mainly the inhibition of electrons flow through the respiratory chain-linked enzymes. Similarly, β -hydroxybutyrate and

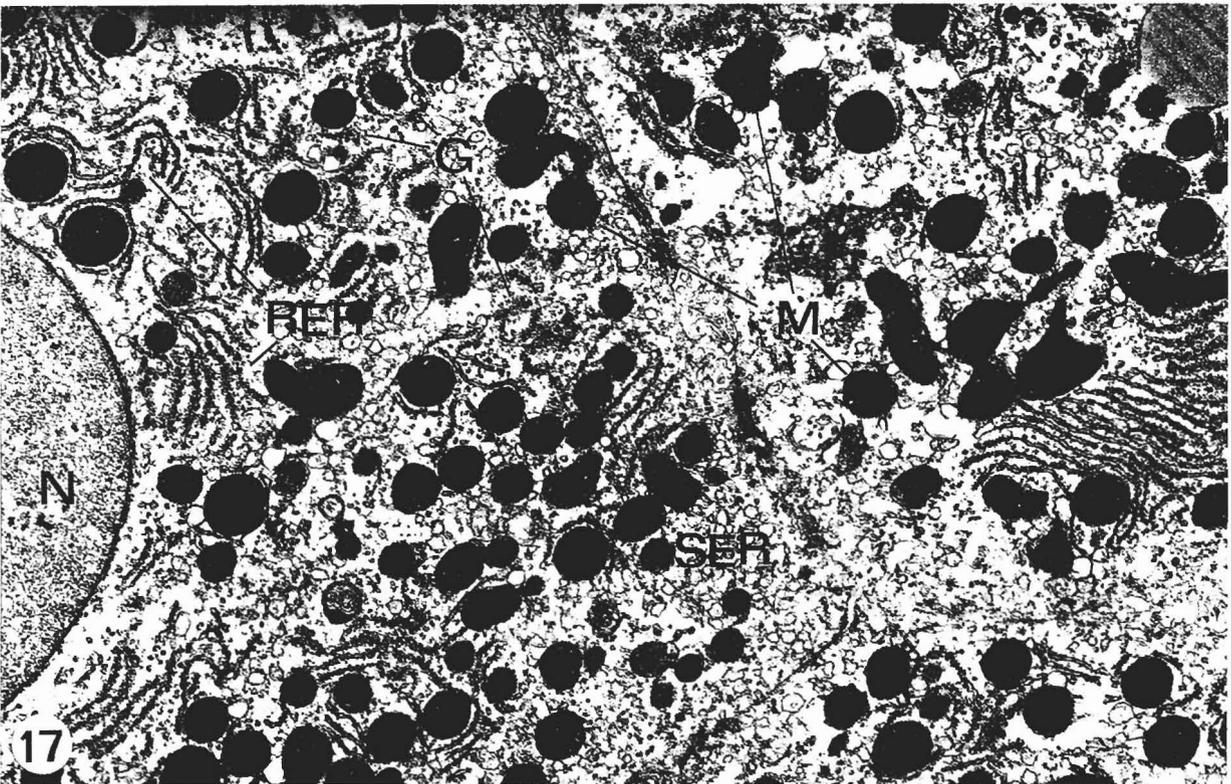
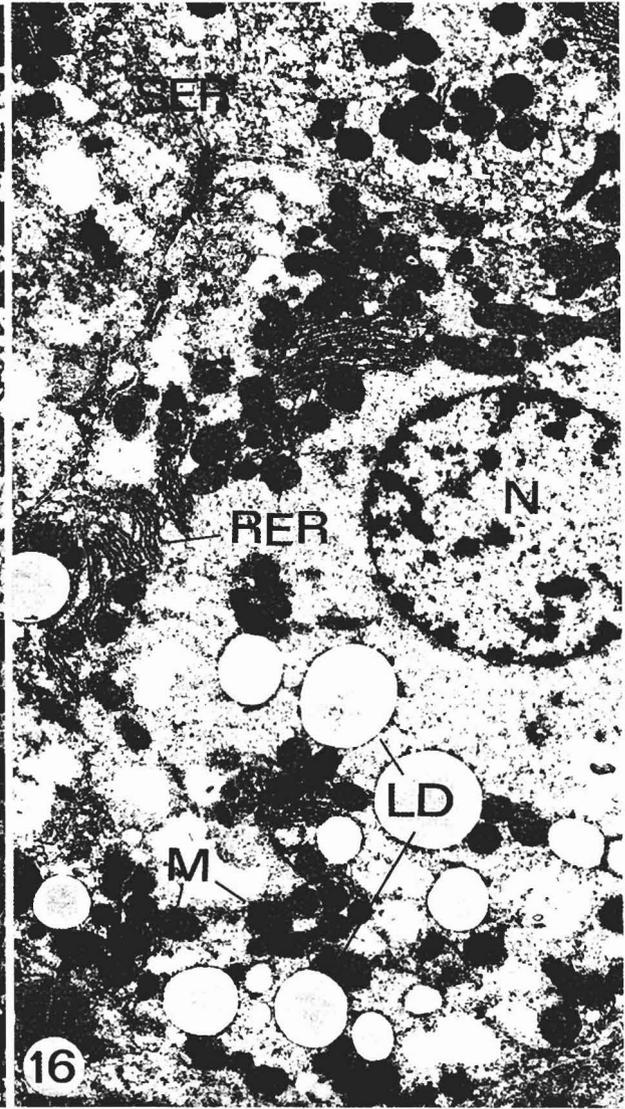
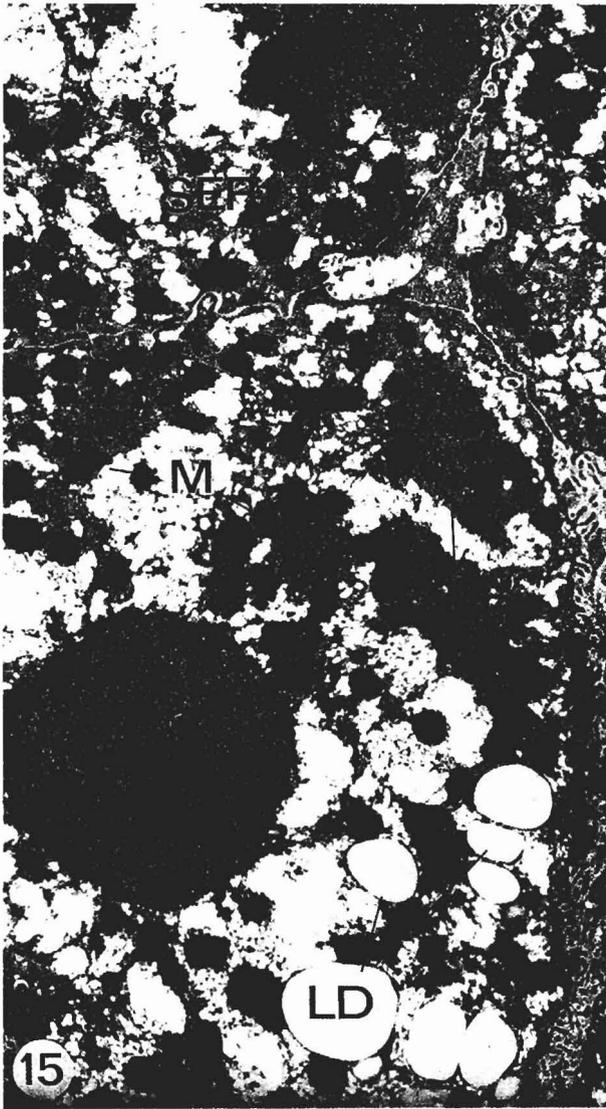
succinate dehydrogenase activities were significantly inhibited by Prudhoe Bay crude oil (Khan et al., 1986). This would inhibit respiration, reduce the amount of ATP for cellular activity thus, resulting in the death of the cell. Fusi et al. (1991), stated that BHT interact with mitochondrial oxidative phosphorylation in two ways: they inhibit electron transport and simultaneously uncouple oxidation from phosphorylation by making the mitochondrial inner membrane permeable to protons. This is in agreement with data reported by Nakagawa et al. (1994). Shertzer et al. (1991) and Thompson and Moldeus (1988) strongly supported that mitochondria represent a major target organelle for BHT toxicity. Shertzer et al. (1991) further suggested that it is difficult to assess lysosomal involvement in the mechanism of cytotoxicity by BHT, although elevation of the lysosomal pH was observed.

Augmentation of lipid droplets in hepatocyte treated with BHT was prevalent, some being fused forming one large globule. When looking for the liver weight gain due to BHT ingestion, one must also answer the following question: does the ingestion of anti-oxidants affect the liver lipid content? A functional explanation for this situation is suggested by the observation that the detoxification of BHT occurs in the liver and that the enlargement of this organ reflects a hyper-functional state resulting from the full effect of this compound. The enlarged liver showed a marked elevation in the concentration of total lipids and esterified cholesterol (Saheb and Saheb, 1977). Thus, it seems that the increases produced by BHT in liver cholesterol occur in parallel with increases in relative liver weight suggesting that both effects might be linked. The formation of lipid droplets, which in turn masked some of the mitochondria after 24 weeks of treatment with 0.2%, 0.4%, and 0.8% BHT are consistent with confirmed findings of increased serum cholesterol and phospholipids in rats administered with BHT (Sahab and Sahab, 1977; Hirose et al., 1981; Takahashi and Hiraga, 1981). Olive oil, when used as a vehicle, for either control or experimental animal groups, may bear some responsibilities for lipid formation in cell cytoplasm. Therefore, when rats were fed on pure or commercial olive oil, it caused a significant increase in lipid droplets in the cytoplasm. Some droplets fused forming larger lipid globules after week 10, as in rats (Akbar, 1997), or after week 12, as in

Fig. 15. Electron micrograph of a portion of liver from rat treated with 0.05% DMBA for 12 weeks. Nuclear damage (N) and severe cytoplasmic disintegration are evident. Proliferated smooth endoplasmic reticulum (SER), and densely stacked rough endoplasmic reticulum (RER) are seen. Mitochondrial alterations are noted, which comprise aberrant shapes; some of them are dumbbell-shaped (arrows). Some mitochondria (M) show complete association with the rough endoplasmic reticulum. Lipid droplets (LD) are quite abundant in the cytoplasm. UA/Pb stain. x 10,000

Fig. 16. Electron micrograph of a portion of liver from rat treated with 3.00% olive oil for 12 weeks. The picture shows the normal structure of hepatocytes. The nucleus (N) is normal. The cytoplasm shows a normal distribution of cell organelles. The smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER) and mitochondria (M) are normal in shape and organization. Many lipid droplets (LD) are abundant. UA/Pb stain. x 8,000

Fig. 17. Electron micrograph of a portion of liver from untreated rat (control). The structure of the hepatocytes is virtually normal. Part of the nucleus (N) is evident. The smooth endoplasmic reticulum (SER) is clearly observed in the cytoplasm. The rough endoplasmic reticulum (RER) and the mitochondria (M) are well developed. Glycogen particles (G) are distributed in the cytoplasm in a normal manner. UA/Pb stain. x 16,000



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mice and monkeys (Nishizumi, 1970) or even after 3 months, as in toad (Sadek and Abdul-Salam, 1994). Also, this observation is in keeping with the findings from animals treated with polychlorinated biphenyl (Nishizumi, 1970; Maclellan et al., 1994a,b) and crude oil (Safer et al., 1997). Schechter et al. (1984), suggested that the increase in cytoplasmic lipid commonly noted in PCB-treated animals, may be attributable to the inability of mitochondria to function properly with respect to the oxidative metabolism normally filled by fatty acids. Takahashi and Hiraga (1981), further stated that BHT significantly decreased the level of non-esterified fatty acids. Our results regarding cytoplasmic lipid augmentation are somewhat at variance with those of Kerr et al. (1966), who failed to observe fat droplets.

Crampton et al. (1977) and Powell et al. (1986), reported that BHT resulted in depression of glucose-6-phosphatase activity, which Feuer et al. (1965), suggested that this phenomenon was greatly accompanied by an increase in the activity of glucose-6-phosphate dehydrogenase. This would be consistent with increased metabolism of glucose via the pentose-phosphate shunt in response to an increased requirement of NADPH to sustain the induced levels of drug metabolizing enzyme activity. This may explain the depletion of glycogen observed in the BHT-treated animals in this study.

Tumorigenicity (hepatocellular adenoma) was not observed in this study, perhaps because it requires a relatively long-term feeding, or could be that the experimental animals used i.e. Sprague-Dawley rats were different than those used by Inai et al. (1988), whom observed neoplastic lesions in the liver of B6C3F1 mice. Moreover, Hirose et al. (1981), found no tumorigenicity when BHT was administered to rats over a 24 month period at concentrations of 0.25% and 0.1%. An increased incidence of liver neoplasia in F1 Wistar rats was also reported, which were delivered from parents administered with BHT for 141-144 weeks. All these liver tumours were found when the F1 rats had survived for more than 2 years (Olsen et al., 1986; Wurtzen and Olsen, 1986).

As a polycyclic aromatic hydrocarbon, DMBA-treated animal in our study, influenced some hepatocytes to loose their nuclei in a dose-dependant manner. However, olive oil-treated animals showed no evidence of ultrastructure abnormalities with respect to control group, except for the presence of lipid droplets and slight to moderate hypertrophy. Similar findings regarding lipid augmentation, has been reported in corn oil-fed rats (Maclellan et al., 1994b,c; Sadek and Abdul-Salam, 1994). In the present study the ultrastructural appearance of hepatocytes from control group were normal.

In considering the results of the present study in terms of the possible human risk of exposure to BHT, it should be emphasized that the average daily intake of BHT in humans is generally far less than the doses used to elicit the liver toxicity in this study (total chemical intake of BHT in this study was several hundred times

higher than the acceptable daily intake). Also, although the cytotoxic effects of BHT in the present study were somewhat in accord with those of DMBA (potent carcinogen), one must consider that the total chemical intake of BHT was several hundred times higher than that of DMBA. Thus, high doses of BHT may be as injurious to the organism as lower doses of more potent chemicals. Bearing in mind that scientists, when conducting a toxicity test of relatively less toxic chemicals in animals, large doses apply (Meyer and Hansen, 1980), while a small dose of radiolabelled compounds is often used when examining the metabolism and disposition (Nakagawa et al., 1979). In addition, the possible cumulative effect of the daily usage of BHT should also be considered when explaining the high dosages applied in this study.

Detailed work are required to show the long-term effect of BHT on hepatocyte to further discover the role of the lysosomal-vacuolar-system in defense against its damaging effects. Also, it is important to perceive the mechanism of mitochondrial transformation from a healthy normal shape to a withered phase with broken cristae.

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