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Ultrastructural changes in rat liver by methyleugenol and evaluation of some biochemical parameters

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Abstract: The present study investigated the effect of methyleugenol, a food flavoring and fragrance agent, on the livers of laboratory rats. Doses of 10 and 30 mg/kg/day of methyleugenol (in 0.5% methylcellulose) were administered intragastrically (IG) to 2 dose groups for 10 days alongside a control group (n = 10 for each group). Gains in body weight were not statistically significant for either dose. Lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) activities decreased ($P < 0.05$) for both dose groups, but AST (aspartate aminotransferase) activity decreased significantly ($P < 0.05$) only in the 30 mg/kg/day methyleugenol dose group. Alanine aminotransferase (ALT) activity did not change significantly in either group. The total amount of glucose remained unchanged, but glycogen in the liver decreased significantly ($P < 0.05$) in the 30 mg/kg/day methyleugenol dose group. There was also an increase of swelling in the smooth endoplasmic reticulum sacs (sER), electron dense accumulations in the sER, cytoplasmic vacuolization, a slight increase of mitochondria and lysosomes, and invaginations in the nucleus membranes of hepatocytes. Extensions in bile canaliculi and increasing microvilli of bile canaliculi in the liver were also observed. These findings indicate the ultrastructural toxic effect of methyleugenol on rat livers in 10 and 30 mg/kg/day doses. Therefore, we may speculate that the toxic effect of methyleugenol needs to be examined in detail.

Key words: Methyleugenol, liver, ultrastructure, ALT, AST, LDH, ASP, glucose, glycogen, rat

Sıçan karaciğerinde metileugenol etkisi ile meydana gelen ince yapı değişiklikleri ve bazı biyokimyasal parametrelerin değerlendirilmesi

Özet: Mevcut çalışmada gıdalar için lezzet ve koku ajanı olan methileugenolün sıçanların karaciğer dokusu üzerine etkileri araştırılmıştır. Metileugenolün 10 and 30 mg/kg/gün dozları (% 0,5 metilselüloz içinde) kontrol grubunun yanı sıra iki doz grubuna intragastrik yolla on gün süreyle verilmiştir (her grup için, n = 10). Vücut ağırlığındaki değişim doz gruplarında istatistiksel olarak anlamlı değildir. Laktat dehidrogenaz (LDH) and alkalin fosfataz (ALP) aktiviteleri her iki doz grubunda ($P < 0,05$) fakat AST (aspartat aminotransferaz) aktivitesi sadece 30 mg/kg/gün methyleugenol grubunda anlamlı şekilde ($P < 0,05$) azalmıştır. Alanin aminotransferaz (ALT) aktivitesi her iki doz grubunda da anlamlı değişiklik göstermemiştir. Karaciğerde total glukoz miktarı değişmeden kalırken, glikojen miktarı 30 mg/kg/gün metileugenol grubunda anlamlı şekilde ($P < 0,05$) azalmıştır. Aynı zamanda, hepatositlerde, düz yüzlü endoplazmik retikulum keselerinde şişme, artış ve elektron yoğun birikimler, sitoplazmik vakuolizasyon, mitokondri ve lizozom sayısında hafif bir artış, nukleus zarında invaginasyon meydana gelmiştir. Ayrıca safra kanalikülilerinde genişleme ve kanaliküllerde microvillus artışı gözlenmiştir. Bu bulgular, metileugenolün 10 and 30 mg/kg/gün dozlarında sıçan karaciğeri üzerine ultrastrüktürel düzeyde toksik etkisine işaret etmektedir. Bu nedenle metileugenolün toksik etkilerinin daha ayrıntılı şekilde araştırılması gerektiğini düşünmekteyiz.

Anahtar sözcükler: Metileugenol, karaciğer, ultrastruktür, ALT, AST, LDH, ASP, glukoz, glikojen, sıçan

Introduction

Methyleugenol is an analogue of the phenolic compound eugenol, and it is a natural constituent of the essential oils of a number of plants widely used in food as flavoring agents (1,2). This alkenylbenzene compound is also used to fragrance cosmetic products such as soaps, shampoos, lotions, and perfumes (3). The reported LD_{50} values of methyleugenol were 850 mg/kg for rats and 540 mg/kg for mice (4). Many biological effects of methyleugenol have previously been reported. Methyleugenol has antibacterial properties (5) and relaxant and antispasmodic actions on the ileum (6). It has been shown to have a depressive effect on the central nervous system with anesthetic, hypothermic, myorelaxant, and anticonvulsant properties (7). Furthermore, methyleugenol has been reported to cause cytotoxicity when measuring lactate dehydrogenase (LDH) release and genotoxicity by using the unscheduled DNA synthesis (UDS) assay in cultured primary hepatocytes of rodents, similar to that caused by safrole or other alkenylbenzene compounds such as eugenol and isoeugenol (3,8). Mutagenicity studies have found that methyleugenol has mutagenic effects in the yeast assay (9,10). Several toxic effects have also been noted, such as hepatocellular injury, altered hepatic function, adrenal hypertrophy at doses of 100 mg/kg or more, reduction of serum total protein and albumin, increases in serum gastrin and gastric pH at doses of 300 or 1000 mg/kg, and increases in serum alanine aminotransferase and sorbitol dehydrogenase activities and bile acid concentration. The no-observed-effect level (NOEL) of methyleugenol for rats and mice was estimated at 10 mg/kg (11). Unfortunately, there are currently no data regarding the toxicity of methyleugenol that include an ultrastructural evaluation. Therefore, we aimed to determine the toxic effect of methyleugenol at 10 and 30 mg/kg/day doses with this ultrastructural and biochemical study.

Materials and methods

Chemicals and dose preparation

Methyleugenol (CAS No. 93-15-2; 99% pure) and methylcellulose (CAS No. 9004-67-5) were purchased from the Merck Chemical Company (Hohenbrunn, Germany) and the Sigma-Aldrich Chemical Company (Steinheim, Germany), respectively. Methyleugenol doses were prepared by diluting the chemical with a 0.5% aqueous methylcellulose suspension, and 2 doses (10 mg/kg/day and 30 mg/kg/day) were administered intragastrically. All other chemicals and reagents used were of analytical grade.

Animals and treatments

Thirty Wistar albino male rats (3 months old, weighing 235 ± 35 g) were obtained from the Department of Experimental Animals of Trakya University, Edirne, Turkey. They were fed standard pellets (including 21% pure protein) and had free access to the water under optimum laboratory conditions (temperature: 21 °C; humidity: 40%-60%; light/dark period: 12 h/12 h; optimum air conditioning system).

Animals were randomly divided into 2 methyleugenol-treated groups and a control group of 10 rats each (n = 10). The first methyleugenol-treated group was given 10 milligrams per kilogram of body weight day of methyleugenol (in 0.5% methylcellulose) and the second group was given 30 mg/kg/day, both by gavage for 10 days. The control group received 0.5% methylcellulose on the same dosing schedule simultaneously. The body weight of the animals was measured and recorded at the beginning and end of the experiment.

Biochemical assay

After 10 days, the animals were anesthetized with 5-10 mg/kg Rompun (2%) (xylazine hydrochloride) from Bayer (İstanbul, Turkey) and 50-70 mg/kg Ketasol (10%) from Richter Pharma (Wels, Austria), and then blood samples were taken from the heart for an enzyme assay (ALT, AST, LDH, and ASP). Enzyme activities were measured using an autoanalyzer (Abbott, Architect C-8000) at Trakya University, School of Medicine, Central Laboratory, Edirne, Turkey.

Liver samples were dissected, washed with cold 0.9% saline, and kept frozen at -80 °C until homogenized. Glucose amounts in the liver tissue were determined following homogenization by a glass-glass homogenizer using the o-toluidine method (12), and glycogen amounts were measured using an

anthrone reagent (13) by spectrophotometer (Shimadzu-1240, UV-VIS).

Histopathological procedures

The animals of each group were killed by decapitation and then the liver specimens of all groups were processed for electron microscopic examination. Liver pieces of 1-2 mm in size were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2-7.3), and, after primary fixations, tissues were washed in 0.1 M phosphate buffer overnight. The tissues were postfixed with 1% osmium tetroxide in phosphate buffer for 1 h at 4 °C. The postfixed tissues were then washed in 0.1 M phosphate buffer and dehydrated using graded ethyl alcohol and, finally, treated with propylene oxide. Dehydrated tissues were processed to make araldite-epon 812 blocks. Ultrathin sections were obtained by ultramicrotome (RMC-MTX Ultramicrotome-USA) and collected on copper grids for double staining (uranyl acetate and Reynolds' lead citrate). Stained sections were observed under a JEOL JEM 1010 transmission electron microscope.

This study was approved by the Local Ethical Committee of Trakya University's School of Medicine.

Statistical analysis

All data were tested for normality using the Kolmogorov-Smirnov test. Normally distributed data were tested using an ANOVA test and then pair-wise comparisons were conducted using Bonferroni's test. Means are given with standard deviations (mean ± SD). $P < 0.05$ was considered statistically significant in the analyses.

Results

Body weight values were not significantly different in the 10 mg/kg/day and 30 mg/kg/day methyleugenol groups compared to the body weight values of the control group. Body weight values are summarized in Figure 1.

Enzyme activities (LDH, ALP, AST, and ALT), as measured by an autoanalyzer, are summarized in Figure 2. It was observed that AST activity decreased significantly ($P < 0.05$) only in the 30 mg/kg/day dose group. LDH and ALP activities decreased ($P < 0.05$) in both the 10 mg/kg/day and 30 mg/kg/day

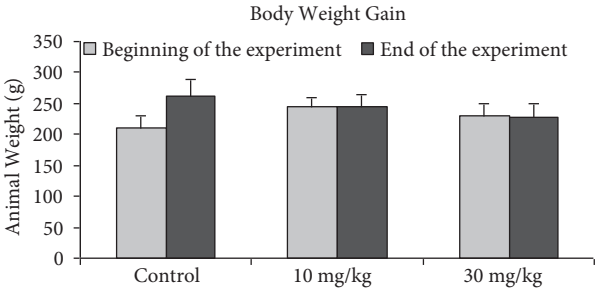

Figure 1. The effect of methyleugenol on body weight (arithmetic mean ± SD; standard deviation is given as an error bar). Body weight values were not significantly different in the 10 or 30 mg/kg/day methyleugenol groups ($P > 0.05$).

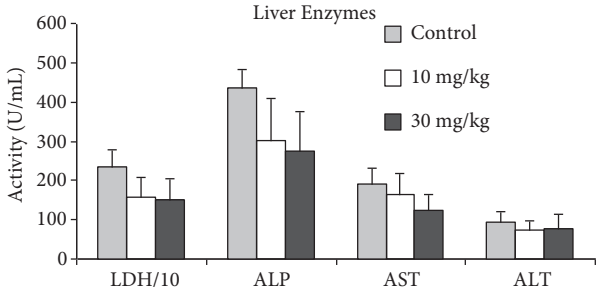

Figure 2. The effect of methyleugenol on LDH, ALP, AST, and ALT. Enzyme activities were measured in serum by using an autoanalyzer (Abbott, Architect C-8000). Decreases in LDH and ALP activities were statistically significant ($P < 0.05$) for both dose groups, but AST activity was statistically significant ($P < 0.05$) in only the 30 mg/kg/day dose group (arithmetic mean ± SD; standard deviation is given as an error bar).

methyleugenol groups. ALT activity did not change significantly for either experimental group in comparison to the control group.

The total amounts of glucose and glycogen are shown in Figure 3. Decreases in glucose were statistically insignificant for both experimental groups, while the amount of glycogen decreased significantly ($P < 0.05$) in the 30 mg/kg/day dose group compared to the control group.

In the electron microscopic observations, the livers of the control group rats were visually normal (Figure 4a). In the 10 mg/kg/day methyleugenol-treated group, we observed an accumulation of electron dense particles in the sER sacs and invaginations in the nucleus membranes (Figure 4b). In addition,

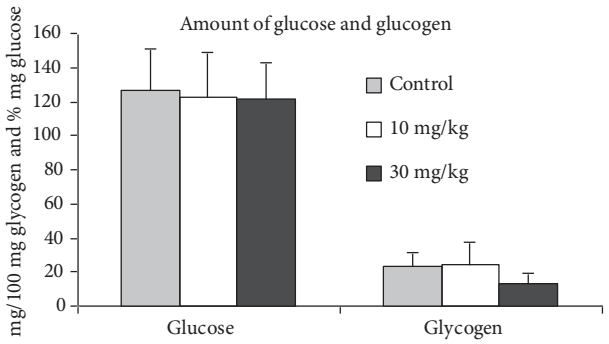

Figure 3. The effect of methyleugenol on the total amount of glucose and glycogen in the liver. Glucose amounts in the liver tissue were determined by using the o-toluidine method (12) and glycogen amounts were measured using an anthrone reagent (13) by spectrophotometer (Shimadzu-1240, UV-VIS). The amount of glycogen was significantly decreased ($P < 0.05$) in only the 30 mg/kg/day dose group (arithmetic mean ± SD; standard deviation is given as an error bar).

cytoplasmic vacuolization, swelling of the sER sacs, and a slight increase in the number of mitochondria were observed in the hepatocytes (Figures 4c and 4d).

In the 30 mg/kg/day methyleugenol-treated group, extensions in bile canaliculi and an increase of microvilli of bile canaliculi (Figure 5a), cytoplasmic vacuolization, swelling of sER sacs, a slight increase in the number of mitochondria (Figure 5b), a decrease in glycogen levels (Figure 5c), and an increase of lysosomes (Figure 5d) were observed in the hepatocytes.

Discussion

Methyleugenol is widely used in food as a flavoring agent (1). However, previous research has been limited to light microscopy and some enzyme activities for an understanding of methyleugenol toxicity. Accordingly, in the present study, we demonstrated dose dependent (10 mg/kg/day and 30

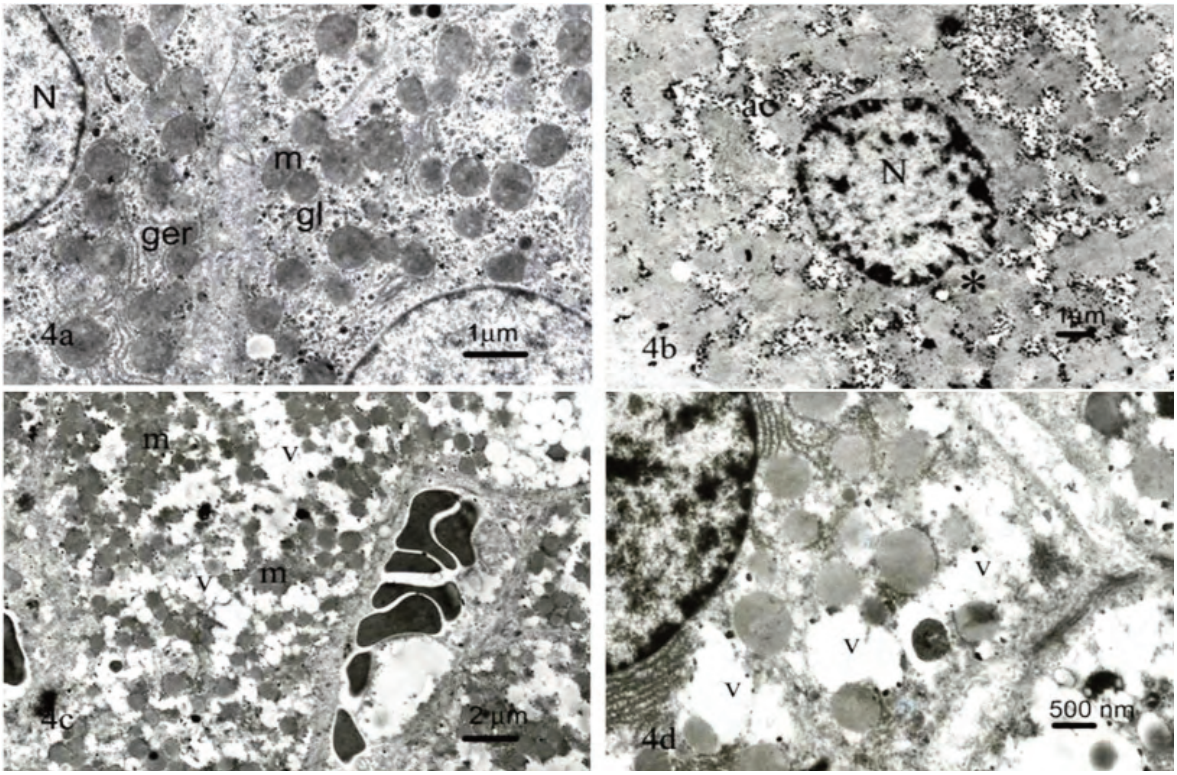

Figure 4. Ultrastructural changes in hepatocytes. a) Control liver. N: nucleus, m: mitochondria, ger: granular endoplasmic reticulum, gl: glycogen. b) 10 mg/kg methyleugenol. ac: accumulation of electron dense particles, invagination of nucleus membrane. c) and d) m: increasing of mitochondria, v: vacuolization. Sections were stained with uranyl acetate and Reynolds' lead citrate.

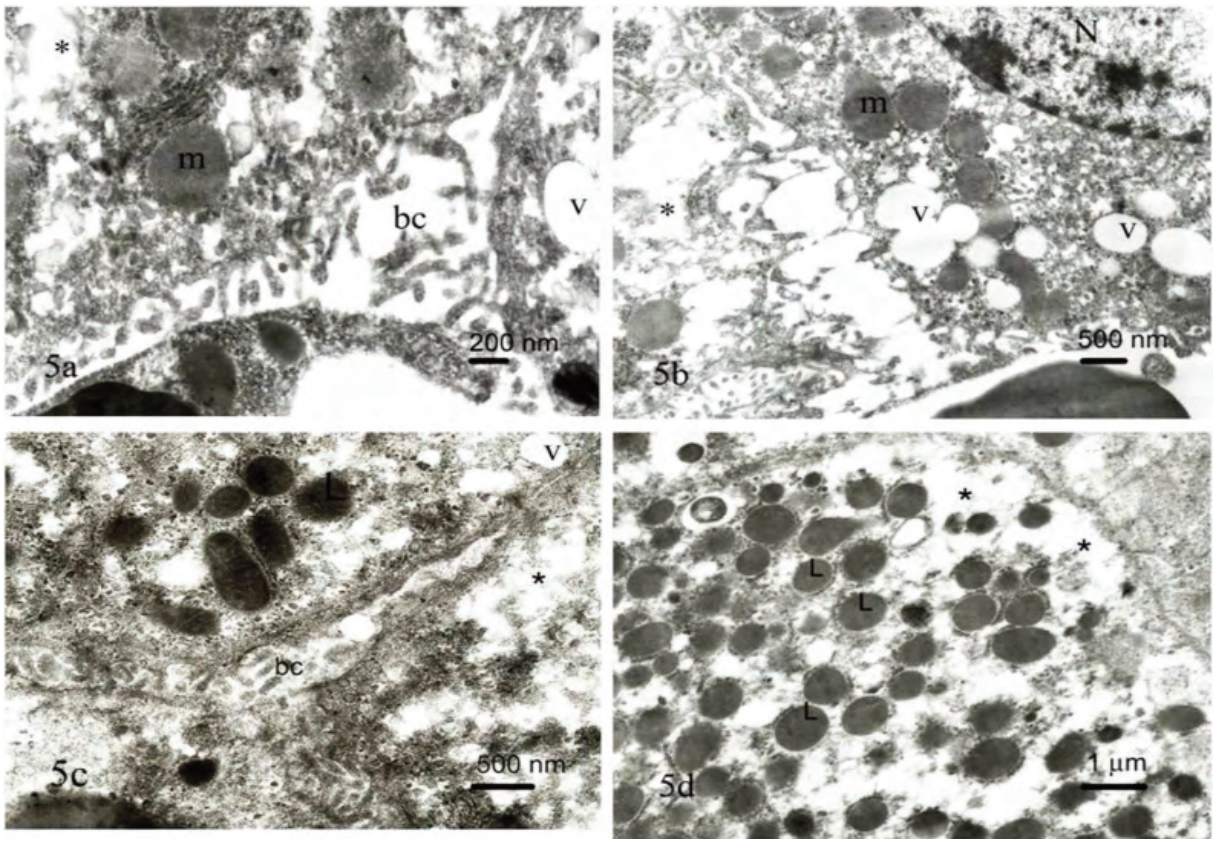

Figure 5. Ultrastructural changes in the 30 mg/kg/day methyleugenol-treated group. a), b), and c) m: increasing of mitochondria, bc: extensions of bile canaliculi, v: vacuolization, N: nucleus. d) *: decrease of cytoplasm, L: increase of lysosomes. Sections were stained with uranyl acetate and Reynolds' lead citrate.

mg/kg/day) methyleugenol toxicity with ultrastructural changes in the liver.

Changes in body weight were not statistically significant for either dose of methyleugenol (10 and 30 mg/kg/day) compared to the control group. In a 91-day dietary study of methyleugenol (daily intake: 18 mg/kg), body weight was not significantly changed (14). In a 90-day gavage study using methyleugenol on mice and rats, the final body weight gains of male rats receiving 300 and 1000 mg/kg/day and for female rats receiving all doses were significantly less than the control group (15). Body weight gains of male and female mice given 300 mg/kg/day were also significantly less than in the control group (15). In our study, the lack of significant weight changes may be explained by the short treatment period and the use of lower doses than other studies.

In a study by Abdo et al., an increase in the ALT serum and a decrease in ALP activity were observed in connection to 100, 300, and 1000 mg/kg doses, and increased sorbitol dehydrogenase activities and bile acid concentrations were observed with 300 and 1000 mg/kg doses of methyleugenol (11). This suggests hepatocellular injury, cholestasis, or altered function in rats. They did not observe any changes in these enzyme activities with 10 or 30 mg/kg methyleugenol doses. Similarly, we did not find any significantly changes in ALT activity with 10 or 30 mg/kg/day doses of methyleugenol in the present study. However, we did observe significantly decreased AST activity in the 30 mg/kg/day dose group. We also found decreases in LDH and ALP activities for both dosage groups. These alterations of liver enzymes may indicate ultrastructural liver injury.

It has been reported that methyleugenol is metabolized by the cytochrome P450 system in the liver (16) and that this metabolism involves O-demethylation, side-chain hydrolysis, and epoxide diol formation (17). 1'-hydroxymethyleugenol and epoxide diol are the main metabolites of this reaction and are responsible for the toxic effect of this chemical (11). We determined slightly increased numbers of mitochondria and decreased amounts of glycogen in hepatocytes by electron microscope, as well as decreased amounts of glycogen with the 30 mg/kg/day dose of methyleugenol. There was no significant change in glucose levels for either dose as measured by spectrophotometer. These findings may be a sign of the metabolizing processes that need energy, and increased energy requirements might be provided by the main energy sources in hepatocytes.

In a 91 day methyleugenol study, normal values were found in a hematological examination, as well as normal morphology for 27 major organs using a histopathological evaluation (14). Previous research has found Kupffer cell pigmentation, bile duct hyperplasia of the liver, and atrophy and chronic inflammation of the mucosa of the glandular stomach with doses of 100, 300, and 1000 mg/kg methyleugenol (11). However, no degenerative changes were found at doses of 10 or 30 mg/kg methyleugenol. Cytologic alteration such as changed tinctorial staining qualities (eosinophilic or basophilic) and increased mitotic activity, and Kupffer cell pigmentation of yellow-gold to green pigment within the cytoplasm of periportal hepatocytes, were characteristic at 100 mg/kg doses and above. Moreover, bile duct hyperplasia, consisting of minimal to moderate proliferation of small bile ductules within portal areas, was observed (11).

In a previous study by the present authors, we also observed some degeneration in the liver, kidney, and small intestine of rats at doses of 10 and 30 mg/kg/day using a light microscope (18). We observed a widening of the sinusoidal area, membrane loss and fusion, cytoplasmic vacuolization in hepatocytes, and degeneration of the endothelium and pyknotic nuclei in the liver. In the present study, we observed increased swelling of the sER and cytoplasmic vacuolization in hepatocytes. These findings are consistent with the light microscopic observations in previous research and may indicate that hepatocytes attempt to metabolize methyleugenol.

We observed an accumulation of electron-dense particles in the sER sacs in hepatocytes of the 10 mg/kg/day dose group. This accumulation may indicate an attempt to metabolize the methyleugenol before the ER induction by the hepatocytes. In our previous study, there was a significant increase in the total amount of protein in the liver, but not in the kidney or small intestine for the 10 and 30 mg/kg/day doses (18). This ER induction and increase in the total amount of protein may be explained as an adaptive response of hepatocytes against methyleugenol.

Invaginations in the nucleus membrane were observed, and this is indicative of a disorder in nucleus metabolism. This may be due to the extension of degeneration of the endomembrane system.

Bile duct hyperplasia of the liver has been reported using a light microscope for doses of 300 mg/kg and above (11). We did not observe any hyperplasia of the bile duct in the 10 and 30 mg/kg/day dose groups by light microscope in our previous study, but, in the present study, extensions in bile canaliculi and increasing microvilli of canaliculi cells in the liver were observed by electron microscope. Bilirubin molecules could not be observed using routine electron microscopy because of their low density (19). However, extensions in the bile canaliculi and the increase in the microvilli of canaliculi cells may be associated with an excessive excretion of bile from the hepatocytes to the bile canaliculi. Additionally, the increase of mitochondria and the decrease of glycogen may corroborate active bilirubin transport, which needs energy. The increase in lysosomes, especially in the 30 mg/kg/day dose group, may be another ultrastructural sign of a response against methyleugenol.

In conclusion, we may speculate that methyleugenol causes liver damage at an ultrastructural level based on our electron microscope observations and the enzymatic evidence for the 10 and 30 mg/kg/day dose groups. Further study, including ultrastructural evaluation, may provide greater insight into the dose dependent toxicity of methyleugenol.

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