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Absence of Acute Testicular Toxicity of Methyl-Tert Butyl Ether and Breakdown Products in Mice

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Methyl Tert-Butyl Ether (MTBE) is a widely used and controversial phytotoxic gasoline oxygenator known to contaminate the environment (Mehlman, 2001; Kampbell et al, 2001; Rousch and Sommerfield, 1998). There is little information regarding its toxicity to animals or its potential effects on reproduction. Concern regarding the possible adverse effects of MTBE prompted its study by scientists from the Toxic Substances Research and Teaching Program (TSR&TP) at the University of California, Davis. The research described here was a part of that research program. Fecal testosterone levels (Billitti et al, 1998), a sensitive non-invasive marker of testicular function, and other relevant reproductive endpoints were used to measure the effects of exposure to MTBE and its environmental degradation products Tert-Butyl Alcohol (TBA) and Tert-Butyl Formate (TBF) (Barreto et al. 1995; Karpel Vel Leitner et al. 1994; Yeh and Novak 1995) in white lab mice (*Mus musculus,* Linnaeus 1766). Levels used were in excess of those to be expected in the environment (Werner *et a*l, 2001).

MATERIALS AND METHODS

Four to 6 month-old CD-1 male mice (Charles River) were kept three to a cage in polypropylene cages (19 x 29 x 13 cm) with food (Purina Mouse Chow) and water available *ad libitum* under a 12 hr light/12 hr dark cycle in humidity (50 ± 10%) and temperature (23 ± 2 °C) controlled rooms. All procedures and animal care were approved by the UCD Animal Care Committee.

After an initial determination of fecal testosterone levels, 20 male mice were randomly divided into four groups and gavaged on days 1, 3 and 5 with 0, 400, 1000 or 2000 mg/kg of MTBE in canola oil. Additionally, three mice were dosed subcutaneously with cadmium chloride ($CdCl_2$), a known testicular toxicant, as a positive control (Billitti *et al.* 1998). Fecal samples were collected on day 6 from all animals before injection with human chorionic gonadotrophin (hCG) to stimulate maximal testosterone production (Billitti *et al.* 1998) and fecal samples were collected at 22 and 26 hours following hCG challenge. After the final collection, blood samples were taken and the testes were removed, weighed and

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preserved in 10% buffered formalin for histopathological examination. Animals dosed with TBA and TBF were gavaged only once, on day 1, and testosterone levels were measured after hCG challenge on day 1 and again on day 4. Two animals died in the 2000 mg/kg MTBE group and two in the 400 mg TBA /kg group as a result of complications from gavaging.

Fecal samples were collected in 30 x 15 cm plexiglass cylinders from individual mice. The bottom of each tube was lined with Whatman 12.5 cm filter paper #4 (Whatman, Maidstone, England) before each sample collection to prevent pooling of urine. After defecation, animals were removed from the chambers, the feces transferred into 1.5 ml flat top microcentrifuge tubes (Fisher, Pittsburgh, PA, USA) and frozen at -20 °C until the day of extraction (Billitti *et al.* 1998) when they were dried, weighed, crushed and extracted for 1 hour with 4 ml of ethyl ether. Water was added (250 µl), partitioning the testosterone into the organic phase, and removed via quick freezing. The extraction was repeated for 30 minutes and the combined extracts evaporated at room temperature and reconstituted in 2 ml of assay buffer (0.1 M $NaPO_4$, 0.1% Bovine Serum Albumin, pH 7.0).

The animals were anesthetized with 200 mg/kg ketamine and 2 mg/kg xylazine in physiological saline and euthanized by cardiac puncture. The blood was collected in 2 ml microcentrifuge tubes and the serum separated according to Goers (1993). A 50 µl serum sample was extracted for testosterone analysis as described above for the fecal samples but with two 2 minute extractions with 300 µl 10% ethyl acetate in pentane (Sigma HPLC grade, St. Louis, MO, USA), and reconstituted in 500 µl buffer.

Testes were prepared for histology as described by Russell (1990). Tissues were sectioned at 3.0 µm and stained with periodic acid-Schiff reagent followed by a hematoxylin counterstain. Histological damage was determined by light microscopic examination of 100-110 seminiferous tubule cross-sections and scored for seminiferous epithelial vacuolization (SEV), marginated chromatin (MC), multinucleated giant cells (MNGC), sloughing and gross disruption (GD) of the seminiferous epithelium (Brown *et al.* 1994).

Testosterone was measured colorimetrically with a competitive heterogeneous ELISA using a polyclonal antibody with high specificity for testosterone (Billitti et al, 1988). Twenty ul of sample were added to a 96 well microtiter plate coated with antibody followed immediately by the HRP-testosterone conjugate. The reaction was run at room temperature for 2 hours or overnight at 4 °C. The plates were washed, a substrate solution of ABTS (2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) and H_2O_2 added and the absorbance measured at 405 nm. Unknowns were compared to a standard curve prepared from 3 to 384 pg testosterone. Detection limit of the ELISA was less than 3 pg/well. Intra- and inter-assay coefficients of variation for the ELISA were 7% and 11%, respectively

(Billitti *et al.* 1998). Chemicals, including steroids, were purchased from the Sigma Chemical Co (St Louis, Mo). Rabbit polyclonal testosterone antibody was produced at UC Davis and provided courtesy of Bill Lasley.

Means, SEM, confidence limits, two-sample t-test, ANOVA, and homogeneity of variance tests were performed using Minitab Statistical Software (Minitab Inc., State College, PA, USA). All graphs were generated with the SigmaPlot Scientific Graphing Program for Windows Version 4.0 (SPSS Inc., Chicago, IL, USA)

RESULTS AND DISCUSSION

MTBE had little effect on testosterone levels and the other reproductive endpoints used in this study, even at high treatment levels. There was no difference in unstimulated or hCG stimulated fecal testosterone at any MTBE treatment level (Figure 1). Endpoint serum testosterone levels did not differ between the untreated control and MTBE dosed animals (Figure 2). Mean body and testes weights also were not different between MTBE dosed and control animals.

There was no difference in the percent change of fecal testosterone or in serum testosterone between the controls and TBA exposed animals. Body weights were increased for all dosing groups due to normal growth. Testis weights of the 1000 and 2000 mg/kg TBA dosed groups averaged 14% higher than the control and 400 mg/kg groups ($p \leq 0.05$). The only significant histological difference was a higher percent of tubules from control animals with sloughing ($7 \pm 2\%$, mean $\pm$ SD, $p \leq 0.05$).

The TBF treated animals had no difference in fecal testosterone levels compared to control animals. End-point serum testosterone of the 1000 mg/kg TBF-treated group was lower than the controls ($p \leq 0.05$), but the 2000 mg/kg TBF treated group was not, indicating the lack of a dose/response relationship. Testis weights of the control group were $22 \pm 11\%$ higher than the three other groups ($p \leq 0.05$), but there were no histological differences between control and 2000 mg/kg exposed animals.

There were no histological differences between control and 2000 mg/kg MTBE exposed animals in the percent of tubules with SEV, MC, MNGC, and sloughing (Table 1). A greater number of tubules showed gross disruption in the 2000 mg/kg group ($6 \pm 2.6\%$, mean $\pm$ SD). No disruption of tubules was observed ($p \leq 0.05$, Table 1) in the control group. (We considered the histopathological damage at the highest dose to be insufficient to warrant examination of the 400 and 1000 mg/kg MTBE groups). In contrast, the CdCl treated positive controls had decreased testis weight, fecal and serum testosterone, and increased histopathological damage compared to the both dosed and control animals ($p \leq 0.05$).

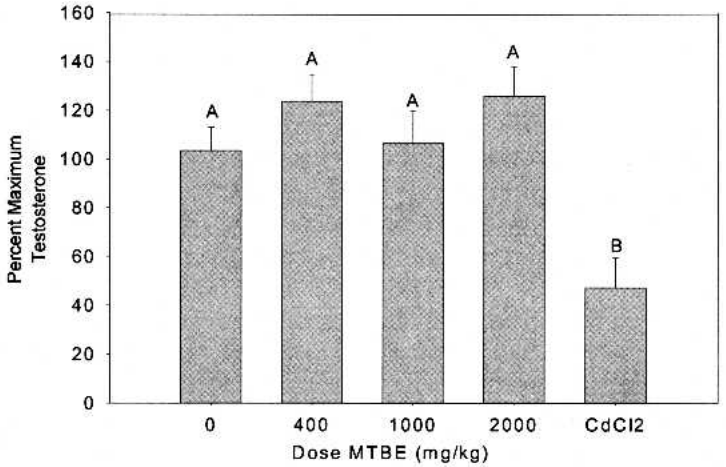

Figure 1. Fecal testosterone levels of mice exposed to 0, 400, 1000 and 2000 mg/kg MTBE with hCG stimulation. Bars represent group means of animals ± SD. N=3 for 2000 mg/kg and $CdCl_2$ groups, all others N=5. Means that do not share the same letter are significantly different ($p < 0.05$, ANOVA).

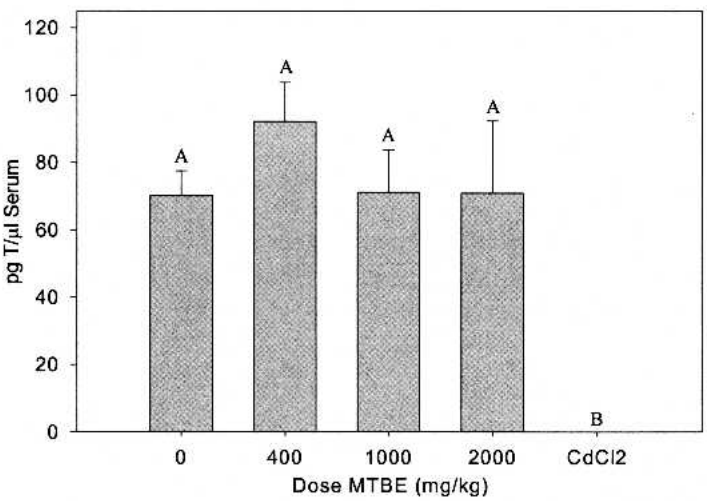

Figure 2. Serum testosterone levels of mice exposed to 0, 400, 1000 and 2000 mg/kg MTBE with hCG stimulation. Bars represent group means of animals ± SD. N=3 for 2000 mg/kg and $CdCl_2$ groups, all others N=5. A: Not significantly different ($p < 0.05$, ANOVA). B. Testosterone was not detected in the $CdCl_2$ positive control group.

Table 1. Histological evaluation of mouse seminiferous tubules after an oral dose of 0.0, and 2000 mg/kg MTBE or an sc dose of $CdCl_2$.

		Percent of Tubules[1]				
	N	SEV[2]	MNGC[3]	MC[4]	Sloughing[5]	GD[6]
Control	3	12.7 ± 4.2	0	0	1.0 ± 1.0	0
MTBE	3	21.3 ± 4.0	0	0	0.7 ± 1.2	6.0 ± 2.6*
$CdCl_2$	3	0.3 ± 0.6	0	0	0	98.3 ± 2.9*

Each value represents the mean percent of tubules ± SD.
When a tubule was noted as grossly disrupted other endpoints were not counted.
[1]Percent of each endpoint was determined with 100-110 tubules/ animal.
[2]seminiferous epithelial vacuolization. [3]multinucleated giant cells. [4]marginated chromatin. [5] sloughing and [6]gross disruption. *Significantly different from control group ($p < 0.05$, t-test).

The results provide evidence that acute exposure to high doses of MTBE and its breakdown products did not cause gross adverse effects on male hormone levels or testicular histopathology.

Reports of effects from MTBE exposure in the humans are sparse; in one report, gasoline vapors containing MTBE were said to cause symptoms consistent with central nervous system (CNS) depression, including headaches, tremors, ataxia, and labored breathing (Mehlman 1996).

In general, the acute toxicity of MTBE is low, with reported LD_{50s} in rats of 4.0 g/kg (oral) and 23,600 ppm (inhalation) (Clayton and Clayton 1981, 1982). More data are available for subchronic and chronic toxicity. In one study, male rats were exposed to 300, 1300, and 3400 ppm MTBE for 6 hours/day, 5 days/week, for 12 weeks, and mated with females that were exposed to similar concentrations for 3 weeks. No differences were observed in several reproductive endpoints, including gonad weights, male accessory reproductive organ weights, organ to body weight ratios, and histopathology (Biles *et al.* 1987). Other studies in mice and rabbits exposed subchronically to MTBE also revealed no observable effects on reproductive health endpoints, including male fertility indices, pregnancy rates, reproduction indices (gestation length, litter size, litter survival), and weights of gonads and organs of the male reproductive tract (Conway *et al.* 1985; Bevan *et al.* 1997). Chronic studies yielded some evidence that MTBE exposure may affect endocrine-sensitive organ systems (Moser *et al.* 1998). Others have demonstrated increased incidence of tumors following chronic inhalation exposure (Belpoggi *et al.* 1995). However, Mennear (1997) has suggested that the report of MTBE-induced tumors is possibly the result of chronic exposure to unrealistically high levels and that such effects should not be extrapolated to the much lower doses to

which humans and other animals are likely to be exposed. In addition, MTBE did not produce point mutations in a *Salmonella* microsuspension assay or clastogenicity in a mouse bone marrow micronucleus test, suggesting a lack of carcinogenic potential (Kado *et al.* 1998).

Although several studies have focused on the potential for reproductive effects of MTBE, until recently no effects on gonad weights, male accessory reproductive organ weights, organ to body weight ratios, histolopathology, male fertility indices, pregnancy rates, gestation length, litter size, and litter survival have been observed by us or by others. However, Almeida and Hall (2004) reported that exposures of mice to 80, 800 and 8000 ppb of MTBE in drinking water for 28 days showed a dose-dependent increase in "mean combined testis weight, mean seminal vesicle weight, mean seminiferous tubule diameter and incidence of abnormal tubules" as well as a decrease in serum testosterone. In addition, high doses of MTBE have been shown to negatively impact offspring, with increased incidences of post implantation loss, altered sex ratios, and reduced mean fetal weights. (Biles *et al.*, 1987; Bevan *et al.* 1997; Conaway *et al.* 1985).

There is little information regarding the effect of TBA on reproduction and carcinogenicity. We also found no published data on reproductive or carcinogenic effects of TBF. Questions remain regarding the effects of chronic exposure to MTBE on reproductive health. The increase in testicular tumors found by Belpoggi *et al.* (1995), suggest the possibility that chronic exposure effects may exist, but questions have been raised about the validity of this study (Mennear 1997). The dose of MTBE was given as a large bolus in a single gavage, which is not a realistic simulation of chronic environmental exposure. Also, the survival of the animals in the high dose group was significantly greater than that of either the control or the low dose group, which is a confounding factor due to the age dependent nature of tumor incidence. In addition, the Belpoggi study and others were run at levels much higher than humans are likely to encounter.

Nevertheless, Mehlman (2001) took strong exception to the National Toxicology Program (NTP) Board, voting 6 to 5 to defeat a motion to list MTBE as "Reasonably anticipated to be a human carcinogen" arguing that the NTP ruling "contravenes rules and procedures previously established...for assessing carcinogenicity..."

Lifetime carcinogenesis studies done under the National Toxicology Program (NTP) guidelines and human epidemiological studies are needed to assess the adverse effects of chronic MTBE exposure. Such studies would help clarify how lifetime exposure to MTBE might adversely affect health.

Immunoassays that utilize serum to measure circulating steroid levels have been an important tool in the field of endocrinology, and the further development of these methods for urinary and fecal steroid measurement makes it possible to non-

invasively study captive and wild animal populations. Though often used to assess the relationships between steroid cycles and mating behavior, the measurement of testosterone in feces also may be a useful tool for monitoring the effects of environmental pollutants on male reproductive function.

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