

Acute Testicular Toxicity of MTBE and Breakdown Products in Lab Mice.

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ABSTRACT

Concern for potential adverse health effects of Methyl tert-Butyl Ether (MTBE) contamination in drinking water have prompted the study of its toxicity by a consortium of University of California scientists. Our laboratory studied the effects of acute oral MTBE exposure on testicular function of male mice using a fecal testosterone ELISA biomarker determined from ether extracts of feces. Baseline and human chorionic gonadotropin (hCG) stimulated levels of fecal testosterone were determined before and after exposure to multiple doses of MTBE. Other parameters examined were animal and testis weights, end point serum testosterone levels, and testis histopathology by light microscopy. Groups of animals (n =5) were orally dosed with 0, 400, 1000 or 2000 mg/kg of MTBE in canola oil three times over a 5 day period. Positive control groups (n =3) were dosed with cadmium (Cd), a known testicular toxicant. On day 7, a final fecal sample was taken, blood was drawn, and testes were removed from each animal. No differences in unstimulated and hCG stimulated fecal testosterone were observed at any treatment level, nor were there any differences seen in endpoint serum testosterone levels($p \leq 0.05$). Body and testis weights were also unaffected by MTBE. The only difference observed in histological evaluation was an increase in gross disruption of tubules in 2000 mg/kg exposed animals ($6 \pm 3\%$, mean \pm SD) compared to controls, in which no disruptions were observed($p \leq 0.05$). In addition to MTBE, we studied two breakdown products produced during UV/peroxidase treatment of water, tert-butyl alcohol (TBA) and tert-butyl formate (TBF). Animals were run through a similar battery of tests as for MTBE. The only endpoint that changed after TBA exposure, was increased testis weight in the 1000 and 2000 mg/kg dosing groups when compared to the 400 mg/kg treated group and control animals. In the TBF treated animals the control animals had testis weights greater than the exposed animals and the 1000 mg/kg group had lower serum testosterone than the control. Overall, the results of this study revealed minimal effects

from acute exposures to high doses of MTBE and its major breakdown products on male hormone levels and testicular histology in mice. However, the potential effects of chronic exposure on reproductive health have yet to be tested.

INTRODUCTION

Methyl tert-Butyl Ether (MTBE) is a widely used oxygenator in gasoline. Despite this, surprisingly few studies have been done to assess the potential adverse consequences of MTBE contamination on human health or the environment. There are reports of exposure to gasoline vapors containing MTBE causing symptoms consistent with central nervous system (CNS) depression, including headaches, tremors, ataxia, and labored breathing (Mehlman, 1996). These acute symptoms are generally not considered to be immediately life threatening, since the acute toxicity of MTBE has been reported to be low. The rat LD₅₀s are 4.0 g/kg (oral) and 23,600 ppm (inhalation) (Clayton and Clayton, 1981-1982). Sublethal and chronic effects of MTBE exposure have not been extensively studied.

There have been several studies of the potential reproductive effects of MTBE. In one study, male rats were exposed to 300, 1300, and 3400 ppm MTBE for 6 hr/day, 5 days/week for 12 weeks, and mated with females exposed to the same concentrations for 3 weeks (Biles et al., 1987). No differences were observed in any reproductive parameters measured, including gonad weights, male accessory reproductive organ weights, organ to body weight ratios, and histopathology. Other studies of reproduction in mice and rabbits exposed subchronically to MTBE have revealed no effects on other reproductive health endpoints, including: male fertility indices, pregnancy rates, reproduction indices (gestation length, litter size, litter survival), weights of gonads and organs of the male reproductive tract (Conaway et al., 1985, Bevan et al., 1997). They did find that higher doses negatively impacted

offspring, with increased incidences of post implantation loss, altered sex ratios, and reduced fetal weight.

Other chronic studies provide evidence of MTBE effects on endocrine-sensitive organ systems and of the potential for carcinogenesis. Moser et al. (1998) found that female mice exposed to a high dose of MTBE vapor had decreased body, ovary and pituitary weights, and prolonged estrous cycles. Belpoggi et al. (1995) exposed rats to MTBE once daily, four days a week, for 104 weeks, and found increased Leydig and interstitial cell tumors of the testes of males and lymphomas and leukemias in females. Despite the finding of increased tumor occurrence after chronic exposure to MTBE, the carcinogenic potential of MTBE is still in question. Mennear (1997) has proposed that MTBE-induced tumors are possibly the result of chronic exposure to toxic doses and should not be extrapolated to the much lower doses to which humans are exposed. Additionally, MTBE did not produce point mutations in a *Salmonella* microsuspension assay or clastogenicity in a mouse bone marrow micronucleus test (Kado et al., 1998).

There is little information regarding the effect of TBA on reproduction and equivocal evidence of carcinogenicity. Increased incidence of renal tubule adenoma or carcinoma (combined) was observed only in male rats when male and female rats and mice were exposed to TBA in drinking water for 2 years (NTIS Publication). However, TBA tested negative in the Ames assay in 4 *Salmonella typhimurium* strains, and in Chinese hamster ovary cells for chromosome aberrations and sister chromatid exchanges (Zeiger et al., 1987, NTP, 1985). No published data on reproductive or carcinogenic effects of TBF was found.

Immunoassays which 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 has made it possible to non-invasively study several captive and wild animal populations. Though often used to assess the relationships between steroid cycles and mating behavior, the measurement of testosterone

in feces may also be a useful tool for monitoring the effects of environmental pollutants, such as MTBE, on male reproductive function. In this study we used fecal testosterone levels, a sensitive marker of testicular function, to measure the effects of oral MTBE exposure on male reproductive function in white lab mice (*Mus musculus*). Testosterone produced by the testis was measured in feces using an enzyme immunoassay developed by our laboratory as a non-invasive biomarker for the effects of pollutants on androgen status in mice (Billitti et al., in press). We also examined the effects of acute oral MTBE exposure on body weight, testis weights, serum testosterone, and testis histopathology. Our goal was to test the hypothesis that acute oral doses of MTBE, TBA, and TBF will alter fecal testosterone levels and the other reproductive endpoints.

MATERIALS AND METHODS

Animals

Experiments were performed on 4 to 6 month-old male white lab mice (*Mus musculus*), purchased from Charles River. Animals were caged three to a cage in polypropylene cages (19 x 29 x 13 cm) with food (Purina Mouse Chow) and water available *ad libitum*. Throughout all experiments, mice were maintained under a 12 hr light/12 hr dark cycle in humidity ($50 \pm 10\%$) and temperature (23 ± 2 °C) controlled rooms. All procedures and animal care were approved by the UCD Animal Care Committee.

Animal Exposures

The testosterone biomarker is determined by measuring ether extracts of feces with an ELISA (Billitti et al., in press). The experimental design involved measuring androgen levels of male mice before and after oral

dosing with MTBE. Measurements of baseline testosterone levels as well as stimulated testosterone levels were made before and after dosing, increasing the sensitivity of the biomarker. Stimulated levels were obtained by subcutaneous (sc) injection of 2.5 IU/g human chorionic gonadotropin (hCG) in 0.9% sterile saline, which stimulates the release of testosterone from the testes (Fail and Whitsett, 1988). After an initial determination of fecal testosterone levels, 23 male mice were divided into five groups. Groups of 5 animals were orally dosed with 0, 400, 1000 or 2000 mg/kg of MTBE in canola oil. Three mice were dosed by injection (sc) with cadmium chloride (CdCl₂), a known testicular toxicant, as a positive control. Animals were dosed three times over the course of 5 days, on days 1,3,and 5. Two animals died from the 2000 mg/kg group during dosing, not as a result of compound toxicity. On day 6, fecal samples were collected from all animals before they were injected with hCG. Fecal samples were collected twice at 22 and 26 hours following hCG challenge. After the final fecal collection blood samples were taken and the testes removed and preserved in 10% buffered formalin for histopathological examination.

Two MTBE breakdown products of UV/peroxidase water treatment, TBA and TBF, were selected for study following a literature search and discussions on MTBE breakdown with Dr. Thomas Young (Yeh and Novak, 1995, Barreto et al., 1995, Karpel Vel Leitner et al., 1994). Animals dosed with TBA and TBF were run through the same battery of tests as MTBE, with the exception that the animals were gavaged only once on day 1, and testosterone levels were measured only after hCG challenge on day 1 and then again on day 4. Two animals died from the 400 mg TBA /kg group during dosing, not as a result of compound toxicity.

Sample Collection and Preparation

Fecal samples were collected from individual mice in 30 x 15 cm (height x diameter) plexiglass cylinders. To prevent pooling of urine, the

bottom of each tube was lined with Whatman 12.5 cm filter paper #4 (Whatman, Maidstone, England) before each sample collection. After defecation, animals were removed from the chambers and the feces were transferred into 1.5 ml flat top microcentrifuge tubes (Fisher, Pittsburgh, PA, USA) and frozen at -20 °C until day of extraction. The samples were extracted using a method previously validated in our lab (Billitti et al., in press). Briefly, fecal samples from the mice were collected, dried, weighed and crushed. Samples were extracted for 1 hour with 4 ml of ethyl ether. Water was added (250 µl), partitioning the testosterone into the organic phase. The organic solvent was removed via a quick freeze technique and evaporated. The process was repeated for another 30 minute extraction. The combined extracts were reconstituted in 2 ml of assay buffer for running on the ELISA.

For blood collection, animals were anesthetized with 200 mg/kg ketamine and 2 mg/kg xylazine in physiological saline, then euthanized via cardiac puncture. The blood was collected in 2 ml microcentrifuge tubes and serum separated using the methods described by Goers (Goers, 1993). A 50 µl serum sample was extracted for testosterone analysis as done for the fecal extraction but with the following changes: It is a 2 minute extraction (2X) with 300 µl 10% ethyl acetate in pentane (Sigma HPLC grade, St. Louis, MO, USA) and the extract is reconstituted with 500 µl buffer.

Testes for histological evaluation were prepared 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 for each testis by light microscopic examination of 100-110 seminiferous tubule cross-sections for seminiferous epithelial vacuolization (SEV), marginated chromatin (MC), multinucleated giant cells (MNGC), and gross disruption (GD) of the seminiferous epithelium (Brown et al., 1994).

Assay

The testosterone was measured colorimetrically using a competitive heterogeneous ELISA previously validated in our lab (Billitti et al., in press). The cross-reactivity of several steroids with the polyclonal antibody shows very high specificity for testosterone. The slopes generated from serially diluted samples to determine parallelism were not different from the slope of the standard curve ($p > 0.1$). The detection limit of the ELISA is less than 3 pg well. The intra- and inter-assay coefficients of variation for the ELISA were 7% and 11%, respectively.

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

RESULTS

There was no difference in either unstimulated or hCG stimulated fecal testosterone at any of the MTBE treatment levels (Figure 1). In addition, there was no difference in endpoint serum testosterone levels between the untreated control and MTBE dosed animals (Figure 2). Body and testis weights were also not different among the MTBE dosed and control animals (Figure 3). Histological evaluation revealed no difference between control and 2000 mg/kg exposed animals in the percent of tubules with SEV, MC, MNGC, and SI (Table 1). There were a greater number of tubules having gross disruption in the 2000 mg/kg group ($6 \pm 3\%$, mean \pm SD) than the controls, in which no disruption of tubules was observed ($p \leq 0.05$). We considered this level of histopathological damage at the highest dosing level to be insufficient to warrant examination of the 400 and 1000 mg/kg MTBE groups due to the time constraints of this study. In contrast, the positive controls (CdCl_2) exhibited decreased testis weight, fecal and serum

testosterone, and increased histopathological damage compared to controls ($p \leq 0.05$).

TBA treated animals showed no difference in the percent change of fecal testosterone (Figure 4). There was also no difference between the controls and TBA exposed animals for serum testosterone (Figure 5). Body weights were increased for all dosing groups due to normal growth. Testis weights of the 1000 and 2000 mg/kg TBA dosed groups were an average of 14% higher than the control and 400 mg/kg group ($p \leq 0.05$) (Figure 6). The only significant histological change was an increase in the percent of control tubules with SI ($7 \pm 2\%$, mean \pm SD), as shown in Table 2 ($p \leq 0.05$).

The TBF treated animals had no difference in fecal testosterone levels compared to control animals (Figure 7). End-point serum testosterone analysis indicated that only the 1000 mg/kg TBF-treated group had levels lower than the controls ($p \leq 0.05$, Figure 8), indicating the lack of a clear dose/response relationship. The testis weights of the control group were $22 \pm 11\%$ higher than the three other groups ($p \leq 0.05$, Figure 9), but histology revealed no differences between control and 2000 mg/kg exposed animals (Table 3).

DISCUSSION

The results of this study did not reveal any dramatic effects of high dose levels of MTBE or its major breakdown products on male hormone levels or testicular histology in mice. This is consistent with the results of acute studies done in rats, which showed no adverse effects of MTBE in male fertility indices, pregnancy rates, reproduction indices (gestation length, litter size, litter survival), weights of gonads and organs of the male reproductive tract (Bevan et al., 1997, Biles et al., 1987). These studies suggest that MTBE should not be a concern for acute toxic effects on male reproduction.

Many questions remain regarding the permanent effects of chronic exposure. It was not possible to study these effects in our study due to time

constraints. The increase in testicular tumors found by Belpoggi, et al. (1995), suggest the possibility that chronic exposure effects may exist, but several questions have been posed 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 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, it is important to note that the Belpoggi study and others were run at levels much higher than humans are likely to encounter. While these are valid criticisms, it does not mean that the results of the study can simply be dismissed. More 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. Further studies will help clarify how lifetime exposure to MTBE affects man.

REFERENCES

- Barreto, R. D., Gray, K. A. and Anders, K. (1995) *Water Research*, **29**, 1243-1248.
- Bevan, C., Neeper-Bradley, T. L., Tyl, R. W., Fisher, L. C., Panson, R. D., Kneiss, J. J. and Andrews, L. S. (1997) *J Appl Toxicol*, **17 Suppl 1**, S13-9.
- Biles, R. W., Schroeder, R. E. and Holdsworth, C. E. (1987) *Toxicology and Industrial Health*, **3**, 519-534.
- Billitti, J. E., Lasley, B. L. and Wilson, B. W. (1998) *Biology of Reproduction*, **In press**.
- Brown, C. D., Forman, C. L., McEuen, S. F. and Miller, M. G. (1994) *Fundam Appl Toxicol*, **23**, 439-46.
- Clayton, G. D. and Clayton, F. E. (1981-1982) *Patty's Industrial Hygiene and Toxicology*, John Wiley Sons, New York.
- Conaway, C. C., Schroeder, R. E. and Snyder, N. K. (1985) *J Toxicol Environ Health*, **16**, 797-809.
- Fail, P. A. and Whitsett, J. M. (1988) *J Androl*, **9**, 21-30.
- Goers, J. (1993) *Immunochemical techniques laboratory manual*, Academic Press, San Diego.
- Kado, N. Y., Kuzmicky, P. A., Loarca-Pina, G. and Moiz Mumtaz, M. (1998) *Mutat Res*, **412**, 131-8.

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- Karpel Vel Leitner, N., Papailhou, A. L., Croue, J. P., Peyrot, J. and Dore, M. (1994) *Ozone Science & Engineering*, **16**, 41-54.
- Mehlman, M. A. (1996) *Toxicol Ind Health*, **12**, 613-27.
- Mennear, J. H. (1997) *Risk Anal*, **17**, 673-81.
- NTP (1985) Review of Current DHHS, DOE & EPA Research Related to Tox, . NTIS Publication No PB96-162748, N. P. N. (No Date) U.S. Department of Health and Human Services, National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709.
- Russell, L. D. (1990) *Histological and histopathological evaluation of the testis*, Cache River Press, Clearwater, Fla.
- Yeh, C. K. and Novak, J. T. (1995) *Water Environment Research*, **67**, 828-834.
- Zeiger, Anderson B, Haworth S, Lawlor T, Mortelmans K and W, S. (1987) *Environmental Mutagenesis*, **9 Suppl 9**, 1-110.

Table 1. Histological evaluation of mouse seminiferous tubules after an oral dose of 0.0, and 2000 mg/kg MTBE or a sc dose of CdCl₂.

	n	Percent of Tubules ¹				
		SEV ²	MNGC ³	MC ⁴	Sloughing ₅	GD ⁶
Control	3	12.7 ± 4.2	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 1.0	0.0 ± 0.0
MTBE	3	21.3 ± 4.0	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 1.2	6.0 ± 2.6*
CdCl ₂	3	0.3 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 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.

¹Percent of each histopathological endpoint was determined with 100-110 tubules from each animal.

²seminiferous epithelial vacuolization

³multinucleated giant cells

⁴marginated chromatin

⁵gross disruption

*Significantly different from control group ($p < 0.05$, t-test).

Table 2. Histological evaluation of mouse seminiferous tubules after an oral dose of 0.0, and 2000 mg/kg TBA or a sc dose of CdCl₂.

	n	Percent of Tubules ¹				
		SEV ²	MNGC ³	MC ⁴	Sloughing ₅	GD ⁶
Control	3	18.0 ± 2.6	0.3 ± 0.6	0.0 ± 0.0	7.0 ± 2.0	0.3 ± 0.6
MTBE	3	20.3 ± 3.8	0.0 ± 0.0	0.0 ± 0.0	1.7 ± 1.2*	0.3 ± 0.6
CdCl ₂	3	1.0 ± 1.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	98 ± 3*

Each value represents the mean percent of tubules ± SD.

When a tubule was noted as grossly disrupted other endpoints were not counted.

¹Percent of each histopathological endpoint was determined with 100-110 tubules from each animal.

²seminiferous epithelial vacuolization

³multinucleated giant cells

⁴marginated chromatin

⁵gross disruption

*Significantly different from control group ($p < 0.05$, t-test).

Table 3. Histological evaluation of mouse seminiferous tubules after an oral dose of 0.0, and 2000 mg/kg TBF or a sc dose of CdCl₂ .

	n	Percent of Tubules ¹				
		SEV ²	MNGC ³	MC ⁴	Sloughing ₅	GD ⁶
Control	3	14.7 ± 2.5	0.0 ± 0.0	0.0 ± 0.0	2.3 ± 0.6	0.7 ± 1.2
MTBE	3	13.0 ± 3.0	0.0 ± 0.0	0.0 ± 0.0	1.3 ± 1.2	1.0 ± 0.0
CdCl ₂	3	0.7 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	98.7 ± 0.6*

Each value represents the mean percent of tubules ± SD.

When a tubule was noted as grossly disrupted other endpoints were not counted.

¹Percent of each histopathological endpoint was determined with 100-110 tubules from each animal.

²seminiferous epithelial vacuolization

³multinucleated giant cells

⁴marginated chromatin

⁵gross disruption

*Significantly different from control group ($p < 0.05$, t-test).

Acute Testicular Toxicity of MTBE

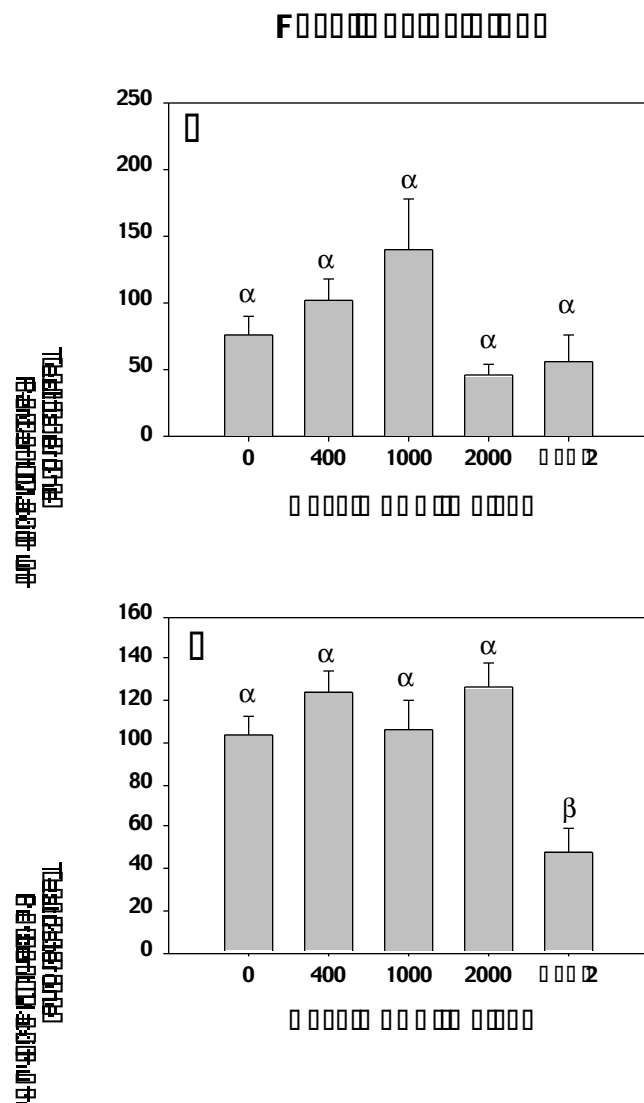


Figure 1. Effect of 0.0, 400, 1000 and 2000 mg/kg MTBE on (A) unstimulated fecal testosterone levels and (B) human chorionic gonadotropin stimulated fecal testosterone levels in mice. Bars represent group means of animals \pm SD. N=3 for 2000 mg/kg and CdCl_2 groups, all others N=5. Means that do not share the same letter are significantly different ($p < 0.05$, ANOVA).

Acute Testicular Toxicity of MTBE

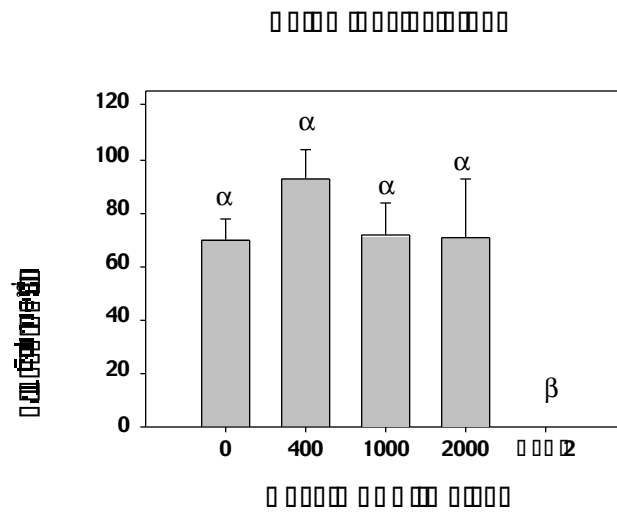


Figure 2. Effect of 0.0, 400, 1000 and 2000 mg/kg MTBE on human chorionic gonadotropin stimulated serum testosterone levels. Bars represent group means of animals \pm SD. N=3 for 2000 mg/kg and CdCl₂ groups, all others N=5. Means that do not share the same letter are significantly different ($p < 0.05$, ANOVA).

Acute Testicular Toxicity of MTBE

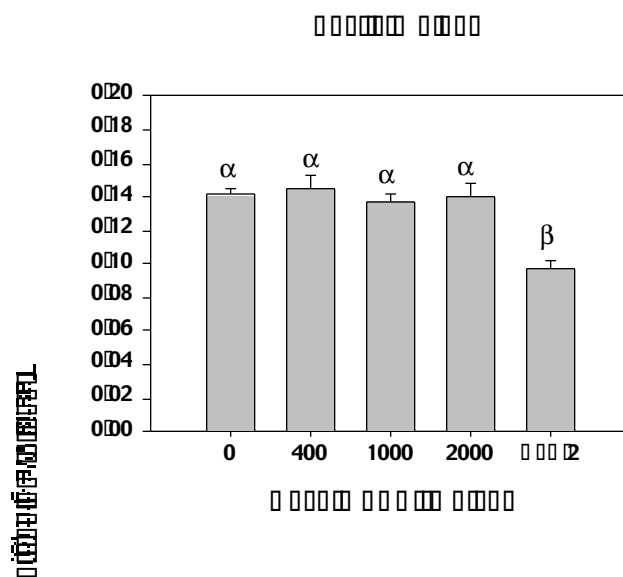


Figure 3. Effect of 0.0, 400, 1000 and 2000 mg/kg MTBE on testis weights of mice. Bars represent group means of animals \pm SD. N=3 for 2000 mg/kg and CdCl₂ groups, all others N=5. Means that do not share the same letter are significantly different ($p < 0.05$, ANOVA).

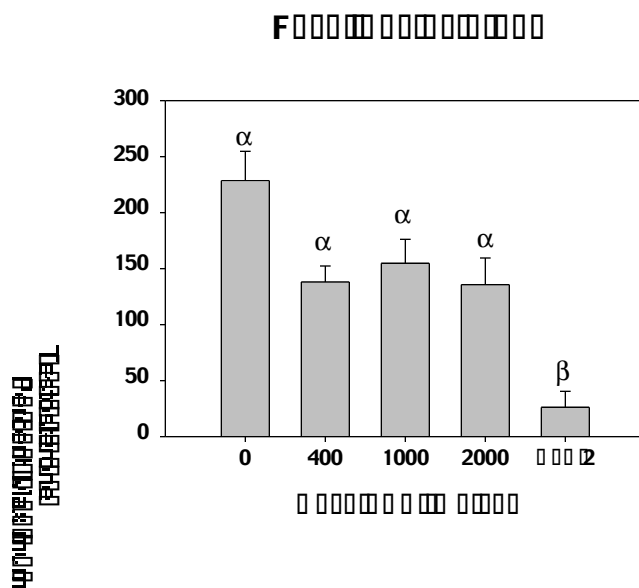


Figure 4. Effect of 0.0, 400, 1000 and 2000 mg/kg TBA on human chorionic gonadotropin stimulated testosterone levels in feces. Bars represent group means of animals \pm SD. N=3 for 400 mg/kg and CdCl₂ groups, all others N=5. Means that do not share the same letter are significantly different ($p < 0.05$, ANOVA).

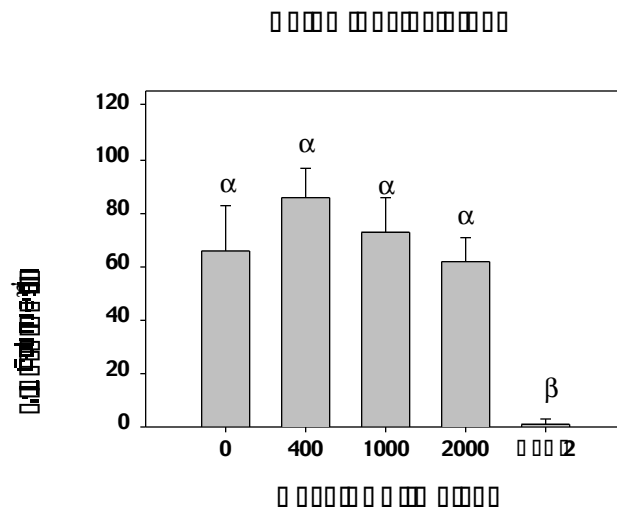


Figure 5. Effect of 0.0, 400, 1000 and 2000 mg/kg TBA on human chorionic gonadotropin stimulated serum testosterone levels. Bars represent group means of animals \pm SD. N=3 for 400 mg/kg and CdCl₂ groups, all others N=5. Means that do not share the same letter are significantly different ($p < 0.05$, ANOVA).

Acute Testicular Toxicity of MTBE

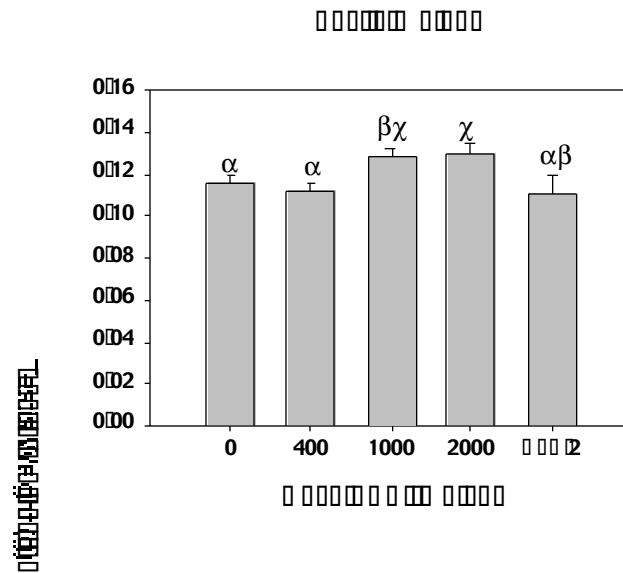


Figure 6. Effect of 0.0, 400, 1000 and 2000 mg/kg TBA on testis weights of mice. Bars represent group means of animals \pm SD. N=3 for 2000 mg/kg and CdCl₂ groups, all others N=5. Means that do not share the same letter are significantly different ($p < 0.05$, ANOVA).

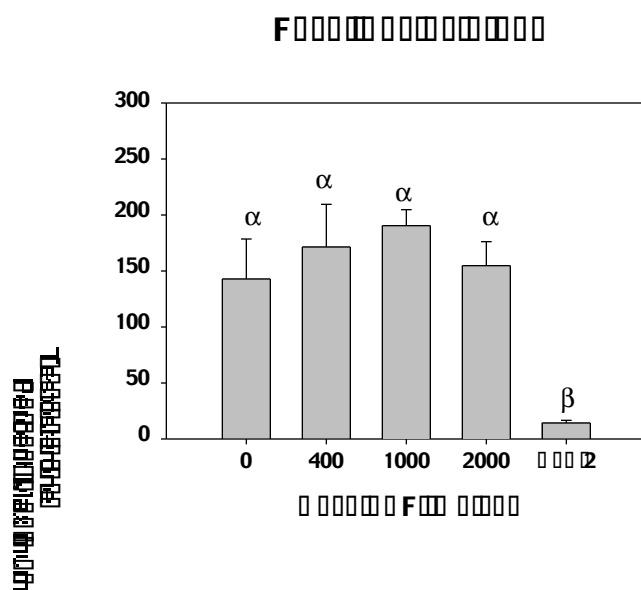


Figure 7. Effect of 0.0, 400, 1000 and 2000 mg/kg TBF on human chorionic gonadotropin stimulated fecal testosterone levels in mice. Bars represent group means of animals \pm SD. N=3 for CdCl₂ group, all others N=4. Means that do not share the same letter are significantly different ($p < 0.05$, ANOVA).

Acute Testicular Toxicity of MTBE

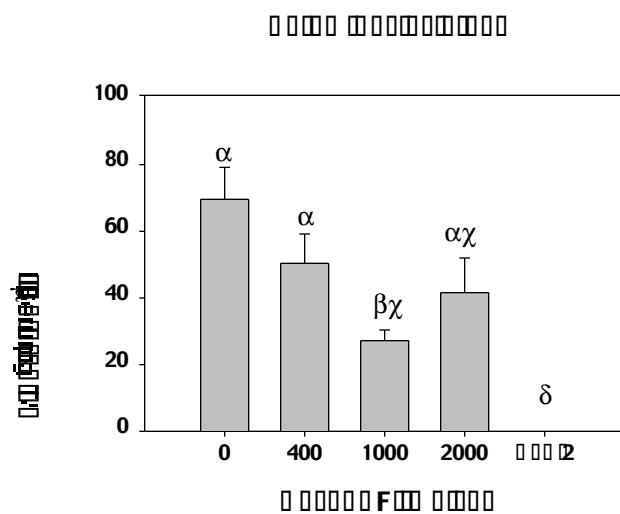
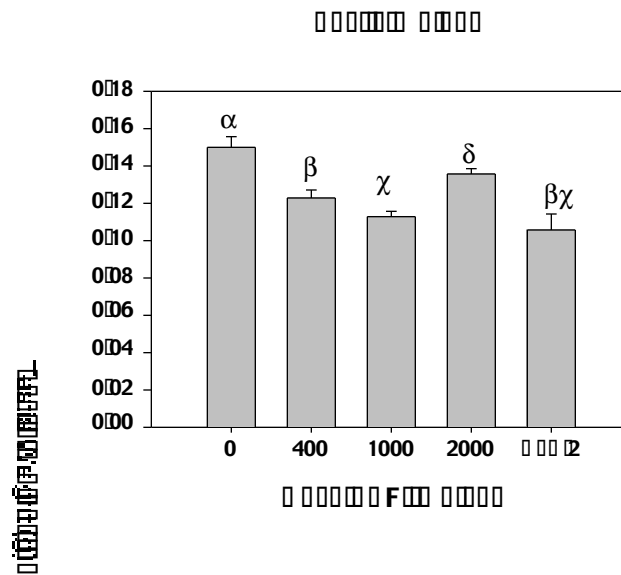


Figure 8. Effect of 0.0, 400, 1000 and 2000 mg/kg TBF on human chorionic gonadotropin stimulated serum testosterone levels. Bars represent group means of animals \pm SD. N=3 for CdCl₂ group, all others N=4. Means that do not share the same letter are significantly different ($p < 0.05$, t-test).

Figure 9. Effect of 0.0, 400, 1000 and 2000 mg/kg TBF on testis weights of mice.



Bars represent group means of animals \pm SD. N=3 for 2000 mg/kg and CdCl₂ groups, all others N=5. Means that do not share the same letter are significantly different ($p < 0.05$, t-test).