Effect of oral methyl-t-butyl ether (MTBE) on the male mouse reproductive tract and oxidative stress in liver

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Abstract

MTBE is found in water supplies used for drinking and other purposes. These experiments follow up on earlier reports of reproductive tract alterations in male mice exposed orally to MTBE and explored oxidative stress as a mode of action. CD-1 mice were gavaged with 400–2000 mg/kg MTBE on days 1, 3, and 5, injected ip with hCG (2.5 IU/g) on day 6, and necropsied on day 7. No effect was seen in testis histology or testosterone levels. Using a similar dosing protocol, others had initially reported disruption of seminiferous tubules in MTBE–gavaged mice, although later conclusions published were consistent with our findings. Another group had also reported testicular and other reproductive system abnormalities in male BALB/c mice exposed for 28 days to 80–8000 ug/ml MTBE in drinking water. We gave these MTBE concentrations to adult mice for 28 days and juvenile mice for 51 days through PND 77. Evidence of oxidative stress was examined in liver homogenates from the juvenile study using MDA, TEAC and 8OH2hG as endpoints. MTBE exposures at the levels examined indicated no significant changes in the male mouse reproductive tract and no signs of hepatic oxidative stress. This appears to be the first oral MTBE exposure of juvenile animals, and also the first to examine potential for MTBE to cause oxidative stress in vivo using a typical route of human exposure.

Keywords

Oral MTBE; male mice; reproductive tract; hepatic oxidative stress

1. Introduction

Methyl tert-butyl ether (MTBE) was adopted as a motor fuel oxygenate in the late 1970s to reduce tailpipe emissions of benzene and other air pollutants (1). Although use as a motor fuel additive has all but been eliminated in the U.S. since concerns arose about

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environmental persistence and increasing presence in soils, groundwater and surface waters, MTBE is still detected in many water supplies in the U.S. and elsewhere that are used for drinking, cooking, bathing, and recreation (2). Uncertainties about the public health implications of chronic exposure to MTBE in water have focused mainly on reports from high dose, lifetime cancer bioassays in laboratory rodents. Cancers reported include increased Leydig cell tumors in rats exposed by inhalation or gavage, hepatic adenomas in female mice exposed by inhalation, and lymphohaematopoetic cancers in female rats treated by gavage (1), yet MTBE currently remains unclassifiable as to carcinogenic potential in humans. This is because of uncertainty about the reliability of the rodent tumor findings, relevance of these tumors seen in only some rodent species to human cancers, and applicability to typically low human exposure levels. For example, it is noteworthy that examination of male reproductive organs after lifetime exposure of rats and mice at identical high inhalation concentrations in the cancer bioassays had revealed Leydig cell adenomas in the testes of rats, but no effect in mouse testes or other reproductive organs. It is not yet known whether rats or mice, or either species, are indicative of human response in terms of health risks associated with MTBE exposure. Whether or not either turns out to be a preferred model for predicting human risk, it is important to be clear on whether effects reported only in brief abstracts can be substantiated.

In subchronic studies focusing specifically on reproductive endpoints no significant effects were reported initially in either male rats or male mice exposed by inhalation. Male CD (Sprague-Dawley) rats were exposed for 12 weeks to up to 3,400 ppm MTBE for 6 hr a day, 5 days a week prior to mating with treated females (3). No adverse effect of treatment was observed in the male reproductive tract, and mating and fertility indices were no different in treated groups compared with air sham controls. No effects were seen in male reproductive organs following MTBE exposure of male rats exposed for 13 weeks or CD-1 mice exposed for 13 days to up to 8,000 ppm (1). A two generation study in CD Sprague-Dawley rats exposed to 8,000 ppm reported reductions of body weight gain early in treatment and in food consumption in the F0 and F1 males but no significant effects on reproductive parameters (4). Oral gavage studies conducted independently by two different laboratories had detected reduced circulating testosterone levels when blood was sampled within 1–2 hr after dosing male Sprague-Dawley rats (5,6). Doses used were high, up to 1,200–1,500 mg/kg, to explore possible mechanisms underlying the rat Leydig cell tumors seen at high doses in cancer bioassays.

Preliminary reports of male mouse reproductive system abnormalities after MTBE orally for up to a month (7,8) were unexpected, however. This is because, unlike the rats, mice in cancer bioassays had no testicular abnormalities after lifetime exposures, and no other subchronic MTBE reproduction studies had reported histopathological changes in the mouse reproductive tract. The first objective of the experiments reported here was to follow up on these two abstracts. Billitti et al. (7) had gavaged CD-1 male mice with MTBE doses of 400, 1000 and 2000 mg/kg on days 1, 3 and 5 of a 7-day study. Almeida et al. (8) had exposed male BALB/c mice through drinking water containing 80, 800 or 8000 ug/L (ppb) MTBE for 28 days. The abstract of the 7-day study had initially reported a small but significant increase in gross disruption of the seminiferous tubules at the 2000 mg/kg dose. After we had conducted a similar follow up experiment reported here and found no effect, the abstract

authors published their study with more details and a final conclusion of no MTBE treatment-related effects (9). The abstract of the 28-day drinking water study had reported an increase in abnormal seminiferous tubules, increased tubular diameters, decreased testosterone, increased testis and seminal vesicle weights, and decreased epididymal weights in animals exposed to MTBE (8). Full details of this study apparently have yet to be published.

A second objective in one of our experiments was to explore the possibility that oxidative stress may play a role in some of the toxic effects seen with MTBE. Previous studies addressing mechanisms underlying MTBE's cancer-causing potential have described evidence of altered endocrine homeostasis (10); however, MTBE could have multiple modes of action. Increased hepatic lipid peroxidation had been reported in 6–9 week old male DDY mice given high intraperitoneal doses of MTBE (11). Glutathione and glutathione-S-transferase, both contributors to the antioxidant protection system of cells, were unaffected in that study. Several groups had investigated the activity of MTBE in Ames Salmonella genotoxicity test strains sensitive to oxidative mutagens. One study reported a weak positive response in Ames Salmonella strain TA102 with an intact excision repair system but not in TA104 lacking excision repair capability (12). Others observed no MTBE effect in TA 104 (13), and no effect of either MTBE or t-butyl alcohol, a metabolite of MTBE, in TA102 (14). MTBE's potential for causing oxidative stress has now also been studied in primary rat spermatogenic cells incubated with 0.5 uM to 5 mM MTBE (15). Reactive oxygen species, membrane lipid peroxidation, and extracellular superoxide dismutase increased, while cytosolic superoxide dismutase decreased. Like the experiments just mentioned, a second paper also appearing in the literature after we had completed our experiments describes other evidence of oxidative stress in male Sprague-Dawley rats (16). In those studies high gavage doses of MTBE ranging from 400 mg/kg to 1600 mg/kg were administered daily for 14 or 28 days. These investigators postulated that other effects they observed in serum LH and FSH--which were sometimes increased and other times decreased relative to controls— were explainable by adjustments in the positive and negative feedback loops present in the complex reproductive endocrine axis to maintain homeostasis. Serum malondialdehyde and total antioxidant ability in serum both increased, while testicular mRNA levels of 8-oxoguanine glycosidase and extracellular superoxide dismutase also increased, all of which suggested increased oxidative stress in rats given high bolus doses. Oxidative stress was interpreted as a possible explanation for the reproductive system changes seen in rats given these high bolus MTBE doses.

MTBE is added to motor fuels to take advantage of its pro-oxidant properties; that is, it provides an additional oxygen source to promote more complete combustion of carbon fuels. The probability of oxygen radical formation increases as oxygen concentration increases, but this does not necessarily mean that oxygen radicals will be formed appreciably in mammalian tissues exposed to MTBE in excess of the body's ability to detoxify them. To test this empirically, we incorporated several measures of oxidative stress into our final experiment.

The 7-day gavage experiment using adult CD-1 mice was patterned after Billitti et al. (7). Two additional drinking water studies consisted of a 28-day drinking water exposure in

adult mice and a 51-day exposure study in younger animals covering the period of weaning to the age of 77–78 days, both using the same mouse strain and MTBE concentrations used by Almeida et al (8). In addition, the livers from mice exposed for 51 days were analyzed for malondialdehyde as an indicator of lipid peroxidation, antioxidant capacity, 8-hydroxy-2'-deoxyguanosine adducts. Our 51-day exposure study appears to be the first oral MTBE exposure of juvenile mice, and the first to explore oxidative stress in animals exposed to MTBE using a typical route of human exposure.

2. Materials and methods

2.1. Chemicals

MTBE (CAS# 1634-04-4) was purchased from Burdick & Jackson (7-day and 28-day exposure studies) or Sigma Aldrich (51-day exposure study). All other chemicals were from Sigma Aldrich or Fisher unless otherwise noted.

2.2. Animals

Adult male CD-1 mice in the one week gavage experiment were purchased from Charles River (Wilmington, MA). Adult male BALB/c mice used in the 28-day drinking water experiment were from Harlan (San Diego, CA). Just weaned juvenile BALB/c mice for the 51-day drinking water exposure study were purchased from Taconic Farms, Inc. (Germantown, NY). All animals were housed in a humidity (55±10%) and temperature (70±4°C) controlled vivarium with a 12:12 light/dark cycle. Caging consisted of polycarbonate shoe boxes with wood chip bedding (Sani-Chips, SaddleBrook, NJ). Mice in the two drinking water experiments were housed singly to allow water consumption measurements for individual animals. Purina 5008 rodent chow was provided *ad libitum* along with tap water initially to all groups during acclimation. The San Diego State University Institutional Animal Care and Use Committee approved all procedures involving live animals.

2.3 Study designs

Approaches, endpoints, tissue preservation methods, analysis kits and other techniques were selected to parallel those used in the earlier 7-day and 28-day mouse studies (7,8) when those details could be determined and were also feasible to replicate. A few exceptions included increasing group sizes in our studies and adding epididymal sperm counts to the 28-day experiment, assuming all deviations from the earlier study protocols could only add more useful information.

7-day gavage experiment—Following the exposure protocol described by Billitti et al., twenty-four adult male CD-1 mice (39–46 g, 96 days at the start of treatment) were randomly assigned to four groups: 0 (corn oil vehicle), 400, 1000 or 2000 mg/kg MTBE (n=6). Similarity of initial group body weight means and standard deviations was verified statistically before finalizing group assignments. Mice were gavaged with MTBE on days 1, 3, and 5, and on day 6 each mouse was injected ip with 2.5 IU/g hCG to stimulate testosterone production. On day 7 mice were anesthetized with a ketamine/xylazine mixture (1 ml/kg of body weight) injected intramuscularly. Blood was obtained by cardiac puncture

before euthanasia by cervical dislocation. Organs were removed and weighed. Blood was placed immediately into Microtainer serum separator tubes, allowed to clot for 40 min, and then centrifuged at 10,000 g for 10 min at room temperature to produce serum for testosterone analysis. Testes were placed in Bouins fixative for 48 hr, then transferred to 70% ethanol for shipment overnight to the histopathology lab.

28-day drinking water experiment—Drinking water concentrations were chosen to replicate the study by Almeida et al. (8). Twenty-four adult male BALB/c mice (n=6), 26–31 g (127 days old when MTBE treatment began), were randomly assigned to the 0 (tap water), 80, 800 and 8000 ppb (ug/L) MTBE experimental groups. Similarity of initial group body weight means and standard deviations was verified statistically before finalizing group assignments. After a 12-day acclimation period, the tap water provided to MTBE treatment groups was prepared to contain these concentrations of MTBE. Water consumption by each mouse was recorded daily. We had also conducted a two week palatability study prior to this using these concentrations of MTBE added to tap water and found no significant differences in water consumption. Food consumption and body weight gain were also not affected during this time by these levels of MTBE. Cage side observations were recorded daily. Euthanasia and serum and other tissue collection were performed as described above for the 7-day study. Testes were placed in 2% glutaraldehyde fixative (Microscopy Science, Hatfield, PA) for histopathology analysis.

Almeida et al. (8) had reported using adult mice; however, we learned later that the age at which these animals had entered their study was 52 days (E. Hall, personal communication), so these mice could still have been just reaching sexual maturity during treatment, whereas our 28-day study mice had begun exposure at a later age. After determining this difference in study design, a third follow up experiment was scheduled to explore whether exposure of younger animals over an extended time period (51 days) might clarify or even intensify the effects reported by Almeida et al.

51-day drinking water exposure of juveniles—BALB/c mice used in this experiment were 22–23 days old (8–11 g) upon arrival. Animals were randomized into four groups (n=10): Tap water control, and those to receive MTBE concentrations of 80, 800, 8000 μg/L when exposure began. Similarity of initial group body weight means and standard deviations was verified statistically before finalizing group assignments. All appeared to be healthy and growing normally after a three day acclimation to the vivarium conditions, food and tap water provided. After a 3-day acclimation, MTBE was added to the drinking water to achieve the nominal concentrations noted above. General health status and amount of water consumed by each mouse was recorded daily. The MTBE exposure period continued for 51 days until sacrifice at which time the mice were 76–77 days of age.

Mice were anesthetized with isoflurane before retro-orbital blood collection and final sacrifice by decapitation. Serum was prepared and aliquoted for hormone assays. Organs of interest were removed and weighed. One testis and associated epididymus were placed in Bouins fixative for 48 hr, then transferred to 70% ethanol before overnight shipment to the histopathology lab. Livers were removed, weighed, and snap frozen after mincing into smaller pieces to promote rapid freezing and facilitate evaluation of these tissues for

evidence of oxidative stress. The frozen liver tissues were sent to the Department of Toxicology at Indiana University for measurement of malondialdehyde as evidence of excessive lipid peroxidation (MDA assay), total antioxidant capacity (TEAC assay), and 8-hyrdoxydeoxyguanine adducts.

2.4 Histopathology of testes and epididymides

Paraffin embedding, slide preparation and histopathology evaluations were completed by Colorado Histo-Prep (Ft. Collins, CO). H&E sections (5 micron) were evaluated for all dose groups in the 7-day gavage exposure experiment, and additional periodic acid/Schiff (PASH)-stained slides were evaluated for samples in the highest dose and control groups. H&E- and PASH-stained testicular sections (5 micron) were evaluated for all mice in the 28-day exposure experiment. In both of these studies one testis was oriented for complete cross section observations and the other for complete longitudinal section. In the juvenile exposure study, complete cross sections were prepared of the testes and both cross sections and longitudinal sections of the epididymides. H&E and PASH sections (5 microns) were evaluated for each mouse. Seminiferous tubule diameters were measured in our laboratory using the H&E-stained slides and an Olympus light microscope with calibrated graticule.

Histopathological lesions considered to be significant were as follows: For testes, tumors, abnormal vasculature, abnormal interstitium, signs of inflammation and presence of large mononuclear or multinucleated cells, mineralization, and within the seminiferous tubules evidence of degeneration, abnormal germinal cell progression, or abnormal Sertoli cells. For epididymides, tumors, abnormal vasculature, signs of inflammation, or abnormal sperm organization.

2.5 Serum hormones

Testosterone Correlate-EIA™ kits (Assay Designs, Inc., Ann Arbor, MI) were used in the 7-day and 28-day exposure studies. Serum was extracted following kit instructions, except that the extraction solvent was 2.0 ml ethyl acetate:hexane (3:2) instead of diethyl ether. Sample and extraction solvent were placed in glass extraction tubes, vortexed for 30 seconds, and then quick frozen in a dry ice/ethanol slurry. The top solvent layer was decanted into a clean glass tube, which was then placed in a water bath at 41.5°C and dried under compressed air flow for 20 min. Samples were then reconstituted in 250 ul of Assay Buffer 3 by vortexing well and allowing to sit and stabilize at room temperature before repeating the extraction. All extracted samples from a given experiment were analyzed in duplicate in a single assay following kit instructions. Typical intra-assay variability was 7.25% (CV) or less. Testosterone and 17β-estradiol were measured in duplicate samples of unextracted serum collected from mice in the juvenile exposure study using ELISA kits not requiring prior extraction and optimized for small sample volumes (Cayman Chemical Company, Ann Arbor, MI). Several repeat assays and sample dilutions were necessary to place all samples within the linear range of the standard curve.

All ELISAs were performed at least twice on aliquots of the same samples on different days to establish that trends observed in mice within a group and relative differences between group means were as shown in the representative assays shown in the tables. Values for

normal control male mouse serum testosterone were expected to be ~10–15 ng/ml based on literature values using radioimmunoassay techniques. To establish accuracy of these kits, commercially available reference control serum samples were analyzed alongside the experimental samples, and these fell within the expected reference control range.

Serum collected after an hCG challenge that was done in the 7-day study to compare with Billitti et al. (7,9) demonstrates potential of the treated mouse testes to respond to an LH-like gonadotropin and to produce and release testosterone. No hCG challenge was done in either the 28-day or 51-day experiments and a similar assay kit was used in order to allow more direct comparison of our serum testosterone results with those reported by Almeida et al. In those experiments, studying non-hCG challenged animals provided a different insight as to whether the entire reproductive endocrine axis still functioned similarly to tap water vehicle controls.

2.6 Sperm counts

The 28-day exposure study endpoints included epididymal sperm counts. After weighing epididymides, the caudae were removed, weighed together and then placed in a Petri dish containing 2.0 ml of phosphate buffered saline (PBS). The two caudae were then minced to release sperm. The resulting suspension was filtered into a clean test tube through 80 micron mesh silkscreen cloth to remove fibrous tissue and other debris from the sperm suspension. An additional 2.0 ml of PBS was then added to dilute the sperm suspension. Each sample was mixed gently before counting sperm cells on a Neubauer hemacytometer. The dose group assignment for each sample was concealed from the scorer until after sperm counts were completed.

2.7 Oxidative stress endpoints

We chose to study lipid peroxidation and other oxidative stress endpoints in liver for several reasons. First, it is widely recognized that MTBE undergoes metabolism in the liver, and this is often the tissue of choice when it is not known whether a metabolite or the parent compound might have this effect. The liver had been used in the only study of lipid peroxidation potential of MTBE in publication at the time our experiments were conducted (11), and we considered analyzing other tissues as well, yet a primary objective was initially to follow up on that study. In addition, substantial tissue mass such as the liver provides was needed for a thorough job with the specific and complementary oxidative stress assays we wanted to use.

Pieces of liver from the juvenile study animals ranging from 0.5 to 1.0 g were thawed and homogenized. Aliquots from each mouse liver sample were designated for total protein, malondialdehyde, Trolox equivalent antioxidant capacity, and 8-hydroxy-2-deoxyguanine adducts. Samples not used immediately remained stored at −80°C. Total protein was determined using a Pierce BCA protein assay kit (Pierce, Rockford IL).

Malondialdehyde (MDA)—The MDA protocol was based on published methods (17,18). A derivative of MDA is formed with 2,4-dinitrophenylhydrazine (DNPH) at low pH. High performance liquid chromatography (HPLC) isolates this specific derivatized MDA analyte

of interest from the complex liver homogenate mixture, and uses UV detection to quantify this derivative. The 1 mM TEP (1,1,3,3-tetraethoxypropane) stock solution used to make an MDA analytical standard was made by acidifying TEP and then diluting immediately before the assay to be completed that day. The rest of the assay was performed as described by Pilz et al. (17) with only minor alterations. A 250 μl aliquot of homogenized sample was mixed with DNPH to form the DNPH derivative of MDA. While protecting the samples from overexposure to UV light after addition of DNPH, each sample was then extracted with 1.0 ml of hexane, then dried down under nitrogen gas. The dried sample was then redissolved in 200 μl of 40:60 acetonitrile:water. The sample was vortexed briefly before loading into the HPLC. HPLC analysis was performed on a Waters Alliance 600S System equipped with a Waters 996 photodiode array detector, a Waters 717 auto injector sampler and Millennium[32] version 4.0 software. Chromatograms were acquired at 290 nm. A Waters symmetry C18 column (3.9 × 50 mm) was used. Elution of the sample was conducted on a linear gradient with a mobile phase A of acetonitrile in water (10:90, v/v) and a mobile phase B of acetonitrile.

Trolox Equivalent Antioxidant Capacity (TEAC)—Changes in the antioxidant defense system of an organism indicate prior exposure to agents causing oxidative damage. The TEAC assay measures the presence of antioxidants in a sample during the generation of the radical cation 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS). The protocol used was a modification of published methods (19). All reagents were purchased from Sigma Aldrich with the exception of ABTS, which was purchased from Fluka. Trolox used as an antioxidant standard in this assay was prepared in phosphate buffered saline, pH 7.4, at a 1/20× dilution. A Tecan Infinite M200 plate reader (Tecan, Durham, NC) with absorbance set at 660 nm was used to quantify antioxidant in the samples, all of which were analyzed in duplicate. A Magellan version 1.0 software system controlled all analytical equipment and carried out the data processing.

8-Hydroxy 2-deoxyguanosine (8OH2dG)—A measure of DNA oxidative damage is the oxidative modification of DNA bases, in particular the C-8 hydroxyl adduct of guanine (20). Of the total oxidized products that are known to occur in DNA, 8-oxo-2'-deoxyguanosine and oxo[8]Gua represent approximately 5% (21). The frequency of these oxidized products and their ability to be measured by electrochemical detection make the 8OH2dG assay a reliable and sensitive measure of oxidative stress-induced changes in the DNA structure. DNA isolation, hydrolysis and analysis of liver homogenates were conducted according to previously published protocols (21,22). All reagents were analytical grade and were purchased from Sigma Aldrich, Fisher or Fluka. HPLC analysis was performed on a Waters 2695 System equipped with a Waters 996 photodiode array detector. The column used with the system was a Supelco LC-18-DB (15 cm × 4.6 cm, 3 μm) with a guard column. 8OH2dG was detected by the ESA CoulArray electrochemical detector set at 100 mV and 400 mV, and 2'-deoxyguanosine (2dG) was detected by the Waters photodiode array detector set at 290 nm. The amounts of 8OH2dG and 2dG in samples were quantitated from their respective calibration curves, and the ratio of 8OH2dG/2dG was compared among different experimental groups to assess the amount of oxidative DNA damage related to treatment.

2.8 Statistical analysis

Body and organ weight, testosterone, sperm count, and oxidative stress measurement data were analyzed using ANOVA. Dunnett's t-test would be applied if between-group differences were significant (p<0.05). Statistical software used was SPSS for Windows version 11 (SPSS Inc, Chicago, IL) for the adult mouse study data and GraphPad Prism 5 for Windows, Version 5.00, March 7, 2007 (GraphPad Software, Inc. San Diego, CA) for the juvenile mouse data.

3. Results

3.1 7-day gavage experiment

Some animals in the 2,000 mg/kg dose group were ataxic and lethargic for up to 60 min each time they were gavaged with MTBE. No significant body or organ weight differences were found at necropsy (Table 1). No noticeable MTBE-treatment related differences were observed in either histological findings in the testes (Table 2) or in group mean serum testosterone which was, as expected, higher in all groups following the challenge dose of hCG than levels found in normal mouse serum. The only testicular abnormality noted in the pathology report was in one control animal with mild unilateral seminiferous tubule degeneration. A mouse in the 8000 ppb MTBE group had an abscess of the preputial gland, a common occurrence in male mice.

Testosterone values showed considerable variability within groups. It was interesting to note in this experiment that a control mouse with unilateral degeneration of the seminiferous tubules, the only testicular abnormality in the pathology report, also had the highest testosterone value measured (68.35 ng/ml). On the other hand, the second lowest testosterone value in this experiment (8.65 ng/ml) was observed in another control mouse with no abnormal testicular histology.

3.2 28-day drinking water experiment in adult mice

Drinking water consumption was similar in all groups for this experiment (Table 3) and mean MTBE consumed was calculated from this. No significant treatment-related differences were seen in group mean body weight or organ weights (Table 4). No clear MTBE dose-related effect was seen in group mean serum testosterone, sperm per mg cauda, or testis histology (Table 5). Minimal or mild degeneration of seminiferous tubules observed in some control mice in addition to treated mice indicated that this was not related to MTBE exposure. All testicular lesions noted were graded as minimal (grade 1) except for one tap water control mouse with unilateral mild degeneration (grade 2).

3.3 51-day drinking water experiment in juvenile mice

All mice remained active and vigorous throughout the exposure period. These MTBE concentrations added to drinking water did not significantly affect average daily water consumption by mice beginning exposure soon after weaning (25–26 days) up to 76–77 days of age (Table 3). Absolute group mean body weights and reproductive and other organ weights were similar across the groups after 51 days of exposure (Table 6). When expressed as relative organ:body weight ratio, the 80 ppb group relative seminal vesicle mean weight

and the 800 ppb group relative lung mean weight were both increased over tap water control weights (p<0.05). This finding did not appear to be related to MTBE exposure insofar as there was no indication of a dose response in the seminal vesicle weights. One mouse in the 800 ppb group had a lung mass that was approximately twice the size of the others in that group, and without that mouse that group relative mean increase would not have been statistically significant. Even still, the tendency toward increasing lung weights as drinking water concentration increased seemed more likely attributable to MTBE exposure. Bloody lung was noted during necropsy for several MTBE treated animals, including the mouse with unusually heavy lungs, and also for one tap water control. Possible underlying causes were not explored further at that time, our main focus being on other organs. No differences were observed in mean seminiferous tubule diameters or abnormal histology in testes or epididymides (Table 7).

Serum testosterone was more variable than expected, possibly because the manufacturer's protocol did not require an extraction step. Whereas eliminating assay steps can sometimes reduce variability, using an assay kit that did not require an additional ether extraction step in the 51-day experiment did not seem reduce within group variability in that experiment, which tended to be even higher than in the 28-day experiment. Inherent biological variability and possibly also interindividual differences in MTBE consumption could also have contributed to variability.

This assay was repeated several times for all samples to confirm that the trends observed in individual mice within a group and between groups was as reflected in the final repeat assay shown in Table 8. Only the lowest 80 ppb group mean was lower than the control mean (not statistically significant). The distribution of lower values than the published normal range (10–15 ng/ml) was approximately the same in all groups: Seven of 10 mice in the control, 800 and 8000 ppb dose groups, and nine of 10 in the 80 ppb group were much lower than in normal sexually mature male mice. Serum estradiol levels were less variable. Neither hormone varied statistically across the groups or suggested a clear dose-response.

Oxidative stress endpoints measured in livers show no evidence of differences resulting from the exposure conditions in the 51-day juvenile mouse experiment (Table 9). The 8OH2dG adducts were measured in one-half of the animals in each treatment group; that is, 20 liver samples of the original 40 samples collected. This assay was completed after the MDA and TEAC assays, and negative results from the TEAC and malondialdehyde assays led to the expectation of minimal if any 8OH2dG level increase. The decision was made then to analyze only the first half of the samples in the more costly 8OH2dG assay. The second half was not analyzed when no group differences were seen.

Taken together, our studies consistently showed no significant differences in male mouse organ weights, testis and epididymal histology, serum testosterone, and caudal sperm counts between the control group and the treated groups with acute gavage exposures up to 2000 mg/kg and subchronic drinking water exposures up to 8000 ppb MTBE in drinking water.

4. Discussion

These three oral dosing studies could not confirm findings of adverse MTBE treatment-related effects on the male mouse reproductive tract noted previously in abstracts by other researchers. By the time our 7-day multiple dosing experiment was completed, Billitti et al. had reevaluated their data and concluded that testis histology and serum testosterone levels were in fact no different from control levels (9), confirming what we had also found using a similar study design.

Our 28-day adult and 51-day juvenile drinking water experiments could not confirm any of the male reproductive tract effects briefly summarized in the abstract by Almeida et al (2). These researchers had observed reduced serum testosterone in the 800 ug/L (ppb) and 8000 ug/L dose groups and reduced epididymal weights at 800 ug/L, but no abnormal morphology in the caput epididymus. Epididymal observations were not reported for the 8000 ppm group. In a separate experiment using the same exposure regimen these researchers had also observed significantly increased combined testes weights in all dose groups (80, 800 and 8000 ug/L) and measurable dose-related increases in both seminiferous tubule diameters and percent abnormal tubules. The reason for different findings in our studies and theirs are not readily apparent. MTBE ingestion in the highest dose groups in our 28-day and 51-day exposure studies was approximately 30 or 40 ug/day/mouse, respectively —slightly higher than the maximum of 22.96 ug/day/mouse reported by Almeida et al. over their 28-day study. The 51-day exposure period of our juvenile mice from PND 25–26 to 76–77 days of age exceeded the 28-day exposure period used by the other group and, as far as we can determine, included the same period of maturation from PND day 52 to PND 80.

In general, testosterone values were more variable than we had expected to see based on extensive prior experience with radioimmunoassays of rat serum. Since high variability can mask differences between groups, experimental and biological variables were considered that could contribute to overall variability in serum testosterone. The ELISA kits used gave us lower testosterone values than we expected, but assay quality control measures employed indicated that the kits were performing satisfactorily. Biological variability may have had a lot to do with variability seen within groups Regularly measuring drinking water consumption by individually caged mice (Table 3) allowed analysis of interindividual differences (coefficient of variation) in mean water consumption per mouse within each group. This was between 6% and 11% in all treatment groups except for the tap water controls in the 28-day experiment, which varied 15%. In the 51-day experiment we suspected that the young age of these mice could also potentially add to the overall variability; that is, mice were just reaching sexual maturity at 51 days, which could result in some individuals either not having acquired the ability to produce stable levels of testosterone, in addition to possibly being more sensitive to MTBE than others.

After no significant effects were seen in the first two experiments, we expanded our focus on the male reproductive tract in the third experiment by including an investigation of the potential for MTBE to cause oxidative stress, applying three complementary measures to all liver homogenates from these male mice. Increased mouse liver tissue levels of MDA were predicted based on findings of a previous report (11). These researchers had conducted acute

ip dosing studies using 6–9 week old male DDY mice: A 50 mg/kg, 200 mg/kg and 500 mg/kg MTBE dose study, and two studies involving one-week and four-week daily dosing with 50 mg/kg and 200 mg/kg. Their single 500 mg/kg dose had reportedly increased lipid peroxidation in liver homogenates 24 hr after dosing but this was not seen 48 hr after this 'one shot' treatment. In their multi-day dosing studies an increase in lipid peroxidation was observed only in the higher, 200 mg/kg MTBE dose group after four weeks of daily ip injections.

Our MDA assay findings indicated no evidence of increased mouse liver lipid peroxidation after 51 days of drinking water containing MTBE and averaging intakes as high as 39.17 ug MTBE/day/mouse. Dividing this average daily intake by the mean final body weight of mice in this experiment (26 g) results in an approximate daily ingestion dose of 1.5 mg/kg/day. Use of a more prolonged and more typical route of human exposure, although with high concentrations of MTBE (up to 8,000 ppm), resulted in daily doses consumed by our mice that were less than 1/10 of the 'one shot' doses used by Katoh et al. (11). High bolus ip doses given to their DDY mice would have resulted in greater peak blood MTBE levels than would be expected with gradual MTBE intake associated with our drinking water ingestion. The increased lipid peroxidation they reported at 24 hr was no longer evident 48 hr after dosing, possibly suggesting a transient effect that could have been mitigated by antioxidant defense and other detoxification and elimination mechanisms of the body. In addition to exposing a younger animal (juvenile vs. adult) and different strain of mouse, we also used a more specific MDA measurement method than was used by Katoh et al. (11). HPLC separation and detection of a fluorescent derivative of MDA provides a more specific and sensitive indication of oxidative breakdown of lipids resulting in accumulation of MDA than the thiobarbituric acid (TBA) colorimetric assay used by Katoh et al (11). The standard TBA assay measures not only MDA but also other TBA-reactive substances present in the sample. A route and dose rate more typical of normal human environmental exposures, and greater specificity in measuring MDA, could alone account for different experimental results. Consistent with the lack of MDA increase we also saw no changes in antioxidant capacity (TEAC) or DNA-altering effect of MTBE in terms of 8OH2dG adducts. Absence of an MTBE effect on 8OH2dG adducts is also consistent with the Ames Salmonella genotoxicity studies finding no evidence of DNA alteration using strains for detecting oxidative mutagens (13,14).

The isolated rat spermatogenic cell exposure experiments demonstrating increases in membrane lipid peroxidation and ROS (15) are more difficult to relate to our lack of oxidative stress response in vivo without knowing blood or testicular tissue levels of MTBE reached in our study. The other group had used a standard, colorimetric TBA reactive-substance assay protocol without prior sample purification, such as more selective method we used involving HPLC, so were not necessarily measuring only the lipid peroxidation MDA byproduct. The continued use of isolated cell cultures to explore the ability of MTBE to affect levels of reactive oxygen species could provide useful information, although keeping in mind that intact organisms have multiple powerful defense systems that protect against xenobiotics causing toxicity through an oxidative stress mode of action. Metabolic activation and detoxification capacities are both especially high in the liver, although both liver and testis contain similar antioxidant defense systems to deal with excess free radicals

generated during P450-mediated steroidogenesis and other metabolic reactions that occur in both organs.

A subsequent in vivo reproductive toxicity publication by Li et al. (16) appeared after all of our experiments were completed showed serum testosterone to be lower in male rats gavaged with high doses of MTBE for 14 or 28 days. Seeing this effect in rats was not surprising to us, since we and others had reported similar testosterone-lowering effects in Sprague-Dawley rats given high bolus doses (5,6). Other effects on rat testes might be expected given the high MTBE doses those rats received. Altered sperm morphology and a trend toward increased epididymal sperm counts with increasing doses after 28 days of MTBE treatment along with disordered seminiferous tubule contents after either 14 or 28 days, had not been reported before, however. In fact, Robinson et al. [23] had observed no treatment-related histopathological findings in testes after 14 or 90 days of gavaging Sprague-Dawley rats with up to 1200 mg/kg MTBE. It should also be noted that Robinson's reported less mortality by the end of their studies than was evident in the data tables presented by Li et al. showing reduced animal numbers in all experimental groups at 28 days.

In addition to focusing on mice rather than rats, and observing no early mortality in any of the experimental groups, our juvenile mouse experiment used a drinking water exposure that was more similar to how humans could be exposed to MTBE. A different protocol would be used if the goal is to determine in mice whether any oral dose of MTBE could cause changes indicative of either oxidative stress or a reproductive hormone imbalance. There are no data from studies in rats using a comparable dosing regimen with which to compare our drinking water exposure testosterone data: Only studies reporting testosterone reduction after high bolus gavage doses. No MTBE+hCG challenge studies that we know of have been conducted using rats. All MTBE studies that we could locate that measured testosterone reported using radioimmunoassays.

Drinking water concentrations used in our studies far exceed current water quality guidelines for MTBE. The range of 80–8,000 ppb MTBE concentrations was approximately 2–200 times greater than the EPA's suggested consumer acceptability level of 20–40 μg/L (4). Our studies to-date reveal no evidence that MTBE alters the male mouse reproductive tract at drinking water concentrations that are much higher than most people would tolerate in terms of taste and odor.

Acknowledgment

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Table 1

Body and organ weights after MTBE gavage exposure: 7-day study in adult male CD-1 mice

	Corn oil control	400 mg/kg MTBE	1000 mg/kg MTBE	2000 mg/kg MTBE
	Absolute weight (g)			
Final body weight	42.3 ± 1.90	41.8 ± 2.21	42.3 ± 2.02	42.2 ± 2.09
Testes	0.255 ± 0.033	0.242 ± 0.023	0.278 ± 0.022	0.248 ± 0.031
Epididymides	0.108 ± 0.008	0.110 ± 0.008	0.111 ± 0.009	0.116 ± 0.006
Seminal vesicles	0.302 ± 0.067	0.321 ± 0.081	0.308 ± 0.037	0.326 ± 0.045
Liver	2.24 ± 0.236	2.26 ± 0.215	2.15 ± 0.281	2.12 ± 0.213
Brain	0.490 ± 0.023	0.507 ± 0.021	0.509 ± 0.226	0.509 ± 0.023
	Ratio of organ:final body weight (%)			
Testes	0.60 ± 0.06	0.58 ± 0.06	0.66 ± 0.05	0.59 ± 0.06
Epididymides	0.25 ± 0.02	0.27 ± 0.02	0.26 ± 0.03	0.28 ± 0.01
Seminal vesicles	0.71 ± 0.15	0.78 ± 0.23	0.73 ± 0.08	0.77 ± 0.08
Liver	5.30 ± 0.55	5.44 ± 0.62	5.08 ± 0.58	5.01 ± 0.39
Brain	1.16 ± 0.04	1.21 ± 0.07	1.21 ± 0.07	1.21 ± 0.06

Notes: Gavage treatment consisted of MTBE in corn oil on days 1, 3, and 5 at doses of 0 (vehicle only), 400, 1000 or 2000 mg/kg MTBE, n=6. 2.5 IU/g body weight hCG was administered ip on day 6 and necropsy was on day 7. Values in the table are mean ± standard deviation. No significant differences were observed across the groups ($p > 0.05$).

Table 2

Testosterone and testis histology after MTBE exposure: 7-day study in adult male CD-1 mice

MTBE treatment +2.5 IU hCG/g	Serum testosterone (ng/ml)[1]	Testis histology
Corn oil control	30.14 ± 21.45	1 of 6 animals had mild unilateral seminiferous tubule degeneration
400 mg/kg MTBE	19.16 ± 7.28	NSL[2]
1000 mg/kg MTBE	27.56 ± 6.47	NSL
2000 mg/kg MTBE	36.08 ± 15.51	NSL

Notes: Gavage treatment consisted of MTBE in corn oil on days 1, 3, and 5 at doses of 0 (vehicle only), 400, 1000 or 2000 mg/kg MTBE, n=6. 2.5 IU/g body weight hCG was administered ip on day 6 and necropsy was on day 7.

[1] Mean ± standard deviation. No significant difference was observed across the groups (p>0.05)

[2] NSL = No significant lesions.

Table 3

Water and MTBE consumption by male BALB/c mice in the two drinking water studies

Group	Adults 28-day exposure (n=6)		Juveniles 51-day exposure (n=10)	
	Mean water consumption (ml/day/mouse)	Mean MTBE consumption (µg/day/mouse)	Mean water consumption (ml/day/mouse)	Mean MTBE consumption (µg/day/mouse)
Tap water control	3.95 ± 0.59	0	4.87 ± 0.35	0
80 ppb MTBE	3.81 ± 0.27	0.305	4.76 ± 0.29	0.381
800 ppb MTBE	3.97 ± 0.29	3.18	4.87 ± 0.47	3.90
8000 ppb MTBE	3.99 ± 0.43	31.92	4.90 ± 0.49	39.17

Notes: Values shown are mean ± standard deviation. Juveniles were exposed from PND 25–26 to PND 76–77. No significant differences were observed in mean water consumption across the groups in either experiment ($p > 0.05$).

Table 4

Body and organ weights after MTBE exposure: 28-day drinking water study in adult male BALB/c mice

	Tap water control	80 ppb MTBE	800 ppb MTBE	8000 ppb MTBE
	Absolute weight (g)			
Final body weight	28.0 ± 1.7	27.8 ± 2.0	28.5 ± 1.1	27.1 ± 2.1
Testes	0.202 ± 0.01	0.208 ± 0.02	0.208 ± 0.02	0.192 ± 0.01
Epididymides	0.073 ± 0.01	0.074 ± 0.01	0.074 ±0.01	0.072 ± 0.01
Seminal vesicles	0.25 ± 0.07	0.23 ± 0.06	0.21 ± 0.05	0.23 ± 0.06
Liver	1.50 ± 0.13	1.48 ± 0.21	1.57 ± 0.09	1.49 ± 0.13
Brain	0.43 ± 0.03	0.44 ± 0.02	0.44 ± 0.04	0.41 ± 0.04
	Ratio of organ:final body weight (%)			
Testes	0.72 ± 0.03	0.75 ± 0.08	0.73 ± 0.04	0.71 ± 0.05
Epididymides	0.26 ± 0.02	0.27 ± 0.03	0.26 ± 0.02	0.25 ± 0.02
Seminal vesicles	0.91 ± 0.25	0.82 ± 0.18	0.73 ± 0.18	0.85 ± 0.16
Liver	5.33 ± 0.34	5.30 ± 0.42	5.48 ± 0.35	5.48 ± 0.19
Brain	1.56 ± 0.12	1.59 ± 0.15	1.56 ± 0.10	1.53 ± 0.14

Note: Values shown are mean ±standard deviation, n=6. No significant differences were observed across the groups ($p > 0.05$).

Table 5

Testosterone, sperm count, and testis histology after MTBE exposure: 28-day drinking water study in adult BALB/c mice

Group	Serum testosterone (ng/ml)[1]	Sperm per mg cauda × 10^5)	Testis histology
Tap water control	2.61 ± 1.56	3.33 ± 0.98	2 mice with unilateral seminiferous tubule degeneration, 1 mild (grade 2), 1 minimal (grade 1)
80 ppb MTBE	2.21 ± 3.42	3.66 ± 1.03	1 mouse with unilateral seminiferous tubule degeneration, minimal (grade 1)
800 ppb MTBE	3.71 ± 7.46	2.79 ± 0.32	1 mouse with unilateral seminiferous tubule degeneration, minimal (grade 1)
8000 ppb MTBE	1.63 ± 0.89	3.06 ± 1.01	2 mice with unilateral seminiferous tubule degeneration, both minimal (grade 1)

Notes: Values are mean ± standard deviation, n=6. No significant differences observed across the treatment groups (p>0.05)

Table 6

Final body and organ weights after MTBE exposure: 51-day drinking water study of juvenile male BALB/c mice

	Tap water control	80 ppb MTBE	800 ppb MTBE	8000 ppb MTBE
	Absolute weight (g)			
Final body weight	26.5 ± 1.40	25.9 ± 1.17	25.2 ± 1.71	25.5 ± 0.610
Testes	0.195 ± 0.011	0.196 ± 0.009	0.188 ± 0.018	0.183 ± 0.019
Epididymides	0.074 ± 0.005	0.075 ± 0.007	0.073 ± 0.007	0.075 ± 0.007
Seminal vesicles	0.169 ± 0.016	0.185 ± 0.026	0.167 ± 0.025	0.165 ± 0.032
Liver	1.41 ± 0.096	1.38 ± 0.118	1.40 ± 0.136	1.41 ± 0.090
Kidneys	0.424 ± 0.037	0.421 ± 0.039	0.411 ± 0.033	0.402 ± 0.033
Brain	0.413 ± 0.018	0.399 ± 0.020	0.401 ± 0.022	0.401 ± 0.021
Spleen	0.100 ± 0.009	0.090 ± 0.010	0.084 ± 0.011	0.089 ± 0.006
Heart	0.145 ± 0.013	0.144 ± 0.017	0.144 ± 0.022	0.137 ± 0.007
Lungs	0.180 ± 0.033	0.191 ± 0.037	0.228 ± 0.072	0.210 ± 0.050
	Ratio of organ:final body weight (%)			
Testes	0.74 ± 0.04	0.76 ± 0.04	0.75 ± 0.07	0.72 ± 0.07
Epididymides	0.28 ± 0.03	0.29 ± 0.07	0.29 ± 0.02	0.29 ± 0.03
Seminal vesicles	0.64 ± 0.09	0.72 ± 0.09[*]	0.66 ± 0.08	0.65 ± 0.13
Liver	5.34 ± 0.23	5.33 ± 0.31	5.55 ± 0.38	5.54 ± 0.31
Kidneys	1.60 ± 0.12	1.63 ± 0.10	1.63 ± 0.11	1.57 ± 0.11
Brain	1.56 ± 0.04	1.55 ± 0.07	1.59 ± 0.10	1.57 ± 0.08
Spleen	0.38 ± 0.02	0.35 ± 0.03	0.33 ± 0.04	0.35 ± 0.02
Heart	0.55 ± 0.04	0.56 ± 0.05	0.57 ± 0.07	0.54 ± 0.03
Lungs	0.68 ± 0.12	0.74 ± 0.17	0.90 ± 0.26[*]	0.82 ± 0.19

Notes: Values are mean ± standard deviation; n=10.

[*] Statistically different from tap water control ($p < 0.05$). Mice were exposed from PND 25–26 to PND 76–77.

Table 7

Seminiferous tubule diameter and histology of testes and epididymides: 51-day study of juvenile male BALB/c mice

Group (n=10)	Mean group tubule diameter (μm)[1]	Histology findings
Tap water control	208 ± 14	NSL[2]
80 ppb MTBE	210 ± 9	NSL
800 ppb MTBE	212 ± 12	NSL
8000 ppb MTBE	208 ± 7	NSL

Notes: Mice were exposed from PND 25–26 to PND 76–77.

[1] Mean ± standard deviation. No significant difference across the groups (p>0.05)

[2] NSL=No significant lesions were noted in any of the H&E- or PASH-stained slides of epididymides or testes. See Materials and methods for explanation of lesions considered significant.

Table 8

Serum testosterone and estradiol after MTBE exposure: 51-day drinking water study of juvenile male BALB/c mice

Group (n=10)	Testosterone (ng/ml)	17β-Estradiol (pg/ml)
Tap water control	7.44 ± 9.94	37.32 ± 8.67
80 ppb MTBE	2.00 ± 3.79	35.89 ± 8.97
800 ppb MTBE	9.83 ± 15.47	36.50 ± 20.29
8000 ppb MTBE	12.50 ± 17.15	32.50 ± 10.10

Note: Mice were exposed from PND 25–26 to PND 76–77. Values in table are mean ± standard deviation; n=10. No statistically significant differences were observed across the groups ($p > 0.05$).

Table 9

Oxidative stress measurements in livers after exposure to MTBE: 51-day drinking water study of juvenile male BALB/c mice

Group	MDA (μM)	MDA (nmol/mg total protein)	TEAC (mM)	TEAC (μmol/mg total protein)	8OH2dG adducts
Tap water control	8.78 ± 2.61	0.424 ± 0.134	4.47 ± 0.43	0.216 ± 0.03	1.378E-05 ± 3.992E-06
80 ppb MTBE	9.61 ± 2.47	0.461 ± 0.131	4.10 ± 0.35	0.200 ± 0.02	1.735E-05 ± 3.421E-06
800 ppb MTBE	8.81 ± 1.67	0.443 ± 0.100	4.29 ± 0.48	0.210 ± 0.03	1.462E-05 ± 3.623E-06
8000 ppb MTBE	8.91 ± 1.30	0.437 ± 0.048	4.10 ± 0.40	0.200 ± 0.03	1.658E-05 ± 4.446E-06

Notes: Mice were exposed from PND 25–26 to PND 76–77.

MDA = malondialdehyde

TEAC = Trolox equivalent antioxidant capacity

8OH2dG= 8-hydroxy-2'-deoxyguanosine; adducts=ratio of 8OH2dG/2dG

Values are mean ± standard deviation; n=10 for the MDA and TEAC assays; n=5 for 8OH2dG adducts.

No significant differences across the groups (p>0.05).