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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 i.p. 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 μg/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.

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 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 lympho-haematopoetic 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 3400 ppm MTBE for 6 h 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

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for 13 days to up to 8000 ppm [1]. A two generation study in CD Sprague–Dawley rats exposed to 8000 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 h after dosing male Sprague–Dawley rats [5,6]. Doses used were high, up to 1200–1500 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 μg/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 μM 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 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 and 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, USA). Adult male BALB/c mice used in the 28-day drinking water experiment were from Harlan (San Diego, CA, USA). Just weaned juvenile BALB/c mice for the 51-day drinking water exposure study were purchased from Taconic Farms, Inc. (Germantown, NY, USA). 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, USA). 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.

2.3.1. 7-Day gavage experiment

Following the exposure protocol described by Billitti et al., 24 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 i.p. 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