Histologic and histomorphometric changes of testis following oral exposure to methyl tertiary-butyl ether in adult rat

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(Received 2 Oct 2014; revised version 1 Feb 2015; accepted 10 Mar 2015)

Summary

Methyl tertiary-butyl ether (MTBE) is used to reduce carbon monoxide and ozone in urban air and to boost fuel octane. There is a lack of knowledge in the literature about the histomorphometric changes of the testis following exposure to MTBE. Therefore, this experimental study was performed to determine the effect of oral exposure to MTBE on histologic and histomorphometric changes of testis in adult rat. A total of 25 adult male Sprague-Dawley rats were randomly divided into five equal experimental groups: control, almond oil and three treatment groups which received 400, 800 and 1600 mg/kg/day MTBE in almond oil by gavages for 30 consecutive days. Histomorphometric analysis showed no significant difference in absolute and relative testis weight, connective tissue thickness, germinal epithelium height, tunica albuginea thickness and Sertoli cell numbers between experimental groups ($P>0.05$). However, trend analysis showed that the seminiferous tubule diameter increased and interstitial cell numbers as well as spermatocyte and spermatid cell numbers decreased significantly in MTBE treated groups ($P<0.05$). It may be concluded that MTBE could exert adverse effects on spermatogenic cells in adult rat. Whether the observed changes in the present study are due to the direct effect of MTBE via passing blood-testis barrier or its indirect effect through another mechanism should be elucidated in future studies.

Key words: Histomorphometric, MTBE, Rat, Testis

Introduction

Methyl tertiary-butyl ether (MTBE) is the main component of oxygenated fuel which has been used worldwide to reduce air pollution, increase the oxygen content of gasoline and decrease the carbon monoxide (Stern and Kneiss, 1997). MTBE can penetrate into the ground and contaminate public water systems. Therefore, people could be exposed to MTBE by swimming, or drinking contaminated water.

MTBE has been listed as a potential human carcinogen (USEPA, 1998). It has been shown to induce interstitial cell tumors of the testes in rats in inhalation and in gavage studies (Belpoggi *et al*., 1995; Bird *et al*., 1997; Clegg *et al*., 1997). In addition to MTBE carcinogenicity, there are other published studies of potential reproductive and endocrine effects of MTBE in male rodents (Day *et al*., 1998, 2000; Billiti *et al*., 2005; Li *et al*., 2008; Williams et Bermudez *et al*., 2012). The results of several toxicity studies suggest that testis is one of the primary target organs in rodents (Williams, 2000; Li *et al*., 2007, 2008, 2009). Despite the testis being a potential target organ for MTBE toxicity, little is known about MTBE-induced histopathological changes in testis.

Li *et al*. (2008) reported an alteration in the testis of high dose MTBE treated groups (800 and 1600 mg/kg/day for 2 and 4 weeks), including an irregular and disordered arrangement of cells in the seminiferous tubules and shedding of cellular material in the seminiferous epithelium in rats.

On the other hand, histomorphometric examination of tissues has a prominent role in male reproductive toxicity evaluation. It could provide information on the severity of toxicity and cellular site of the damage. To the best of the authors knowledge, there is no study concerning the histomorphometric evaluation of testis following exposure to MTBE. Therefore, the objective of the present study was to determine the effect of oral administration of MTBE on histological and histomorphometric changes of testis in adult Sprague-Dawley rats. In this study, by quantification of changes in MTBE exposed testis at the light microscopic level, we aimed to determine the possible adverse effects of MTBE on the reproductive system in this animal model.

Materials and Methods

A total of 25 adult Sprague-Dawley male rats 7-8 weeks old and weighing 223 ± 20 g were purchased from animal house of "Sepid Exir Azma", Shiraz, Iran. Animals were housed in stainless steel cages under standard animal house conditions with a 12 h light/dark cycle and a temperature of 25 ± 2°C, received standard pellet food, and tap water was available *ad libitum*. The experimental animals were randomly divided into five equal experimental groups after 10 days of acclimation period: control, almond oil and three treatment groups which received 400, 800 and 1600 mg/kg/day MTBE in

almond oil by gavages for 30 consecutive days. MTBE was prepared from Oil Refinery, Shiraz, Iran with 98.8% purity. The study was approved by our institutional review board. Body weight and group food consumption were measured every week. In 800 mg/kg/day MTBE group, one animal expired due to inappropriate gavages before the end of the study.

At the end of the exposure period, male rats were anesthetized with ether. Their testes were removed, testicular weight was recorded and they were then fixed in 10% formalin for 48 h. After processing for paraffin embedding, serial sections with 5 µm thickness were stained with haematoxylin and eosin and used for histological and histomorphometric studies at the light microscopic level. Seminiferous tubule diameter, germinal epithelium height, the connective tissue thickness and tunica albuginea thickness were measured using the Axiovision L.E. 4.5 software. For measuring seminiferous tubule diameter, 90 round or nearly round cross-sections of seminiferous tubules were randomly chosen in each rat. Two perpendicular diameters of each cross-section of seminiferous tubules were measured using an ocular micrometer of light microscopy (Olympus EH) at magnification of ×40 and their means were calculated. Also, germinal epithelium height was measured in 4, equidistance of each cross-section of the seminiferous tubules and their means were calculated. For measuring the number of Interstitial cells, Sertoli cells and spermatogenic cells, Image tools 3 software was used. In each rat, six sections and 5 fields per section (in other words, 30 fields in each rat) per unit level (1 mm^2) were randomly counted at magnification ×10 of light microscopy (Olympus EH).

Data were presented as mean, median and standard deviation. All measured variables were compared between groups using nonparametric analysis of variance (Kruskal-Wallis test) followed by Mann-Whitney U test due to non-normal distribution of data and low number of samples in each group. Jonckheere's analysis was used to determine their dose-response trend. Changes in body weight and food consumption during the study period were analyzed using repeated measures analysis of variance. Data were analyzed using SPSS statistical software (Version 16.0; SPSS, Inc., Chicago, USA) and a p-value less than 0.05 was considered statistically significant in all analyses.

Results

Mean body weight was increased from the start of the study toward the end of the experiment ($P<0.001$) with no statistical difference between experimental groups ($P=0.40$). Average food consumption was 169 ± 32 g in the first week and increased to 349 + 28 in the last week of the study ($P<0.001$). No significant difference was detected for absolute ($P=0.75$) and relative ($P=0.28$) testis weight (g) between experimental groups (Table 1).

Histological studies showed no lesions in control and almond groups (Figs. 1a-b). In all MTBE treatment groups, changes were observed in seminiferous tubule as pyknosis, decreased cell layers, increased cell distance as well as the presence of vacuoles in the epithelium (Figs. 2a-c). The severity of lesions enhanced with increased dose of MTBE. The results of the statistical comparison of histomorphometric variables is presented in Tables 2 and 3. No significant changes were observed for connective tissue thickness ($P=0.48$), germinal epithelium height ($P=0.32$) and tunica albuginea thickness ($P=0.95$) in controls compared with MTBE treated groups. However, seminiferous tubule diameter showed significant increase in MTBE treated groups compared with almond oil group ($P=0.023$, Table 2). On the other hand, results showed a decrease in interstitial cell numbers as well as spermatocyte and spermatid cell numbers in MTBE treated groups in comparison with control ones ($P<0.05$). Spermatogonia ($P=0.15$) and Sertoli cell numbers ($P=0.98$) did not show significant changes (Table 3). When trend analysis using Jonckheere's test was performed, results showed that there are significant decreasing trends for number of cells including interstitial ($P=0.002$), spermatogonia ($P=0.024$), spermatocyte ($P=0.001$) and spermatid ($P=0.001$) in the experimental groups. For spermatogenic cells, this trend is depicted in Fig. 3. The increasing trend

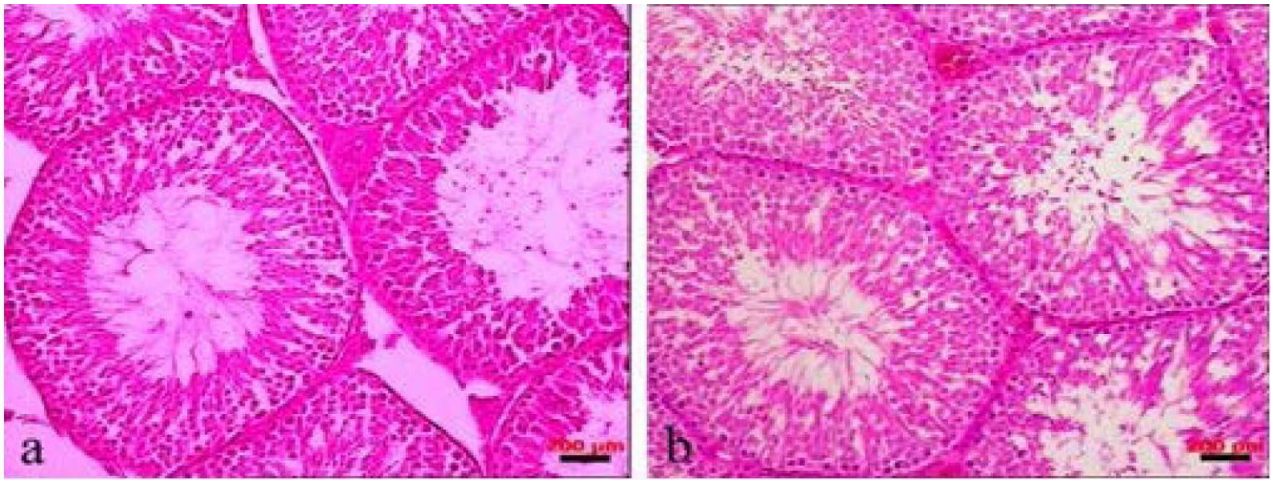

Fig. 1: Histological section of adult rat testis in experimental groups. a: Control, and b: Almond oil (scale bar 200 µm, H&E)

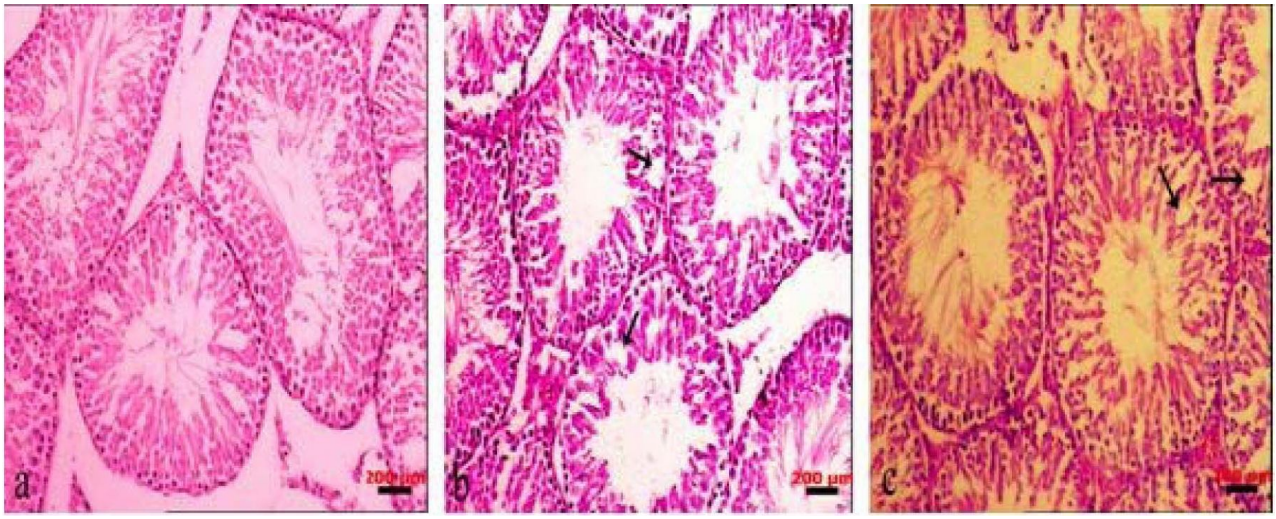

Fig. 2: Histological sections of adult rat testis in treatment groups. a: 400, b: 800, and c: 1600 mg/kg/day MTBE. In Figs. 2b and c, arrowheads indicate presence of vacuoles in epithelium (scale bar 200 µm, H&E)

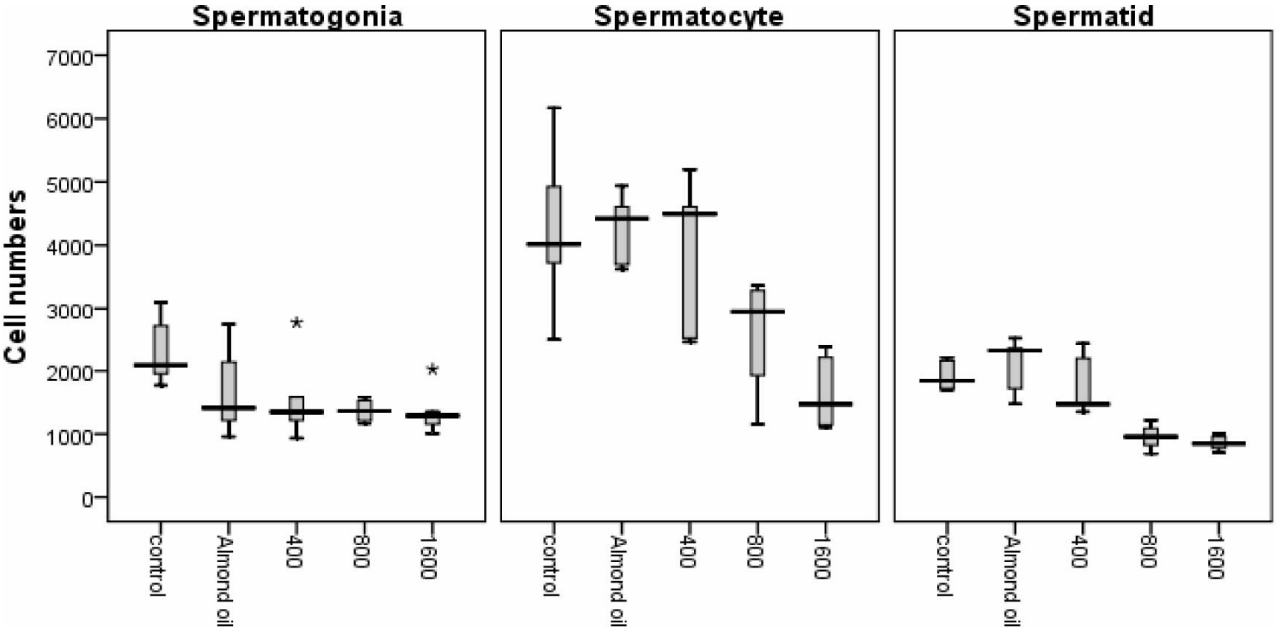

Fig. 3: Box plots showing minimum, maximum and quartiles of number of cells of testis in controls and MTBE (mg/kg/day) treated groups in adult Sprague-Dawley rats. Asterisks show extreme values

Table 1: Comparison of testis and body weights (g) in MTBE (mg/kg/day) treated groups with control groups in adult Sprague-Dawley rats

Experimental groups		Absolute testis weight	Relative testis weight	Final body weight
Control	Mean	1.20	0.004	267.40
	Std. deviation	0.45	0.001	44.27
	Median	1.00	0.004	295.00
Almond oil	Mean	1.60	0.006	271.60
	Std. deviation	0.89	0.003	33.15
	Median	1.00	0.004	276.00
400 mg	Mean	1.40	0.005	303.80
	Std. deviation	0.55	0.002	5.21
	Median	1.00	0.003	300.00
800 mg	Mean	1.25	0.004	291.75
	Std. deviation	0.50	0.001	19.77
	Median	1.00	0.004	292.50
1600 mg	Mean	1.60	0.006	272.40
	Std. deviation	0.55	0.002	25.08
	Median	2.00	0.006	265.00

No statistical difference was observed between groups (P>0.05)

Table 2: Comparison of thickness (T), diameter (D) and height (H) of various parameters in testis of MTBE (mg/kg/day) treated groups with control groups in adult Sprague-Dawley rats

Experimental groups		Tunica albuginea T.	Connective tissuse T.	Seminiferous tubules D.*	Germinal epithelum H.
Control	Mean	42.37[a]	46.59[a]	289.91[ab]	98.49[a]
	Std. dviation	15.49	8.33	57.62	8.37
	Median	43.09	44.80	284.84	97.95
Almond oil	Mean	42.08[a]	50.86[a]	267.92[b]	104.71[a]
	Std. deviation	3.12	10.53	21.70	5.03
	Median	42.1500	46.94	263.14	105.47
400 mg	Mean	41.77[a]	53.24[a]	319.78[ab]	110.65[a]
	Std. deviation	6.57	10.16	29.32	16.29
	Median	39.97	56.20	321.24	109.49
800 mg	Mean	42.62[a]	53.04[a]	342.62[a]	109.83[a]
	Std. deviation	6.67	13.24	25.99	6.78
	Median	42.74	58.64	341.65	111.63
1600 mg	Mean	41.93[a]	60.86[a]	345.78[a]	110.30[a]
	Std. deviation	9.44	14.81	38.90	12.01
	Median	37.26	60.19	320.70	109.96

Different superscript letters in each column show significant differences (P<0.05). * Show significant increasing trend by Jonckheere test (P<0.05)

Table 3: Comparison of number of cells in testis of MTBE (mg/kg/day) treated groups with control groups in adult Sprague-Dawley rats

Experimental groups		Number of cells				
		Sertoli	Interstitial *	Spermatogonia *	Spermatocyte *	Spermatid *
Control	Mean	44.58[a]	61.50[a]	2327.16[a]	4270.56[ab]	1928.64[a]
	Std. deviation	6.765	8.733	559.527	1375.386	245.139
	Median	49.20	61.50	209.10	61.50	49.20
Almond oil	Mean	44.28[a]	54.12[a]	1694.94[a]	4263.18[a]	2081.16[a]
	Std. deviation	10.947	16.482	732.096	1112.658	454.731
	Median	36.90	61.50	1414.50	4428.00	2324.70
400 mg	Mean	44.28[a]	49.20[a]	1571.94[a]	3859.74[ab]	1788.42[a]
	Std. deviation	6.765	15.00	708.726	1277.97	493.476
	Median	49.20	49.20	1353.00	4501.80	1476.00
800 mg	Mean	43.05[a]	43.05[a]	1371.45[a]	2610.675[bc]	956.325[b]
	Std. deviation	7.134	7.134	193.356	157.686	215.988
	Median	43.05	43.05	1365.30	2958.15	959.40
1600 mg	Mean	41.82[a]	39.90[b]	1367.76[a]	1665.42[c]	858.54[b]
	Std. deviation	14.02	8.733	392.985	599.994	127.059
	Median	36.90	36.90	1291.50	1476.00	848.70

Different superscript letters in each column show significant differences (P<0.05). * Show significant decreasing trend by Jonckheere test (P<0.05)

for seminiferous tubule diameter was observed, too (P=0.003).

Discussion

In the present study using histomorphometric evaluation of testis, we observed that oral administration of MTBE could exert adverse effects on spermatogenesis process and cause some histological changes. Control groups showed a compact and regular arrangement of spermatogenic cells in the seminiferous tubules whereas in all MTBE treatment groups, deceased cell layers and increased cell distance were observed. This is in agreement with the study of Li *et al*. (2008) who showed that seminiferous tubules of rats in high dose MTBE treated groups had less impact cells and irregular and disordered cell arrangement. Also, Billiti *et al*. (2005) evaluated the acute effects of oral MTBE exposure on testicular function in male CD-1 mice. Histopathological examination showed a slight increase in the number of tubules with gross disruption in the 2000 mg/kg group.

Decreasing trend in the number of spermatogonia, spermatocytes and spermatids (Fig. 3) indicated that all germinal cells are influenced by MTBE, although the effect is more prominent in the more mature cell types i.e. spermatocytes and spermatids, and in higher dose groups. One simple explanation for this finding may be the indirect effect of decrease in the serum testosterone level in MTBE treated animals which has been reported by previous studies (Williams *et al*., 2000; de Peyster *et al*., 2003; Li *et al*., 2008). Furthermore, reduced number of interstitial cells in MTBE treated groups which are

testosterone secreting cells in the male reproductive system could be considered supporting evidence for the aforementioned finding.

In contrast to the significant decline in spermatogenic cell numbers, no significant change was observed in the germinal epithelium height of the seminiferous tubules. This finding could be attributed to the disordered arrangements in the cells, so that lowering cell numbers was compensated by the increasing distance between cells.

We observed that Sertoli cell numbers were not changed following MTBE exposure. Sertoli cells are supportive cells of the testis and their tight junctions are responsible for the formation of blood-testis barrier. Li *et al*. (2007, 2009) conducted a series of *in vitro* experiments and reported that MTBE exerts toxic actions directly on spermatogenic and Sertoli cells in culture. They suggested that exposure to MTBE *in vivo* could impair spermatogenesis through toxic actions exerted directly on spermatogenic or Sertoli cells. Taken together, whether the observed changes in the number of spermatogenic cells in the present study are due to the direct effect of MTBE via passing blood-testis barrier or is an indirect effect through another mechanism such as hormonal changes should to be elucidated in the future studies.

It is worth noting that a few of the previous studies on MTBE evaluated the testicular histopathology, with the majority reporting no effect (Bird *et al*., 1997; Williams *et al*., 2000; de Peyster *et al*., 2008; Bermudez *et al*., 2012). We suppose that the major reason for the observed difference in our results with the previous ones is the qualitative approach of evaluation in all previous studies compared with the quantitative way in ours. We also suggest that assessment of changes at the electron microscopic level be conducted in the future studies in this field.

Acknowledgements

The authors would like to thank Shiraz University for financial support and Dr. F. Namazi, pathologist for her assistance to this work.

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