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Oxidative DNA Damage Induced by Toluene is Involved in its Male Reproductive Toxicity

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Toluene is widely used as an organic solvent in various industries and commercial products. Recent investigations have shown that toluene may induce male reproductive dysfunctions and carcinogenicity. To clarify whether the toxicity results from the interference of endocrine systems or direct damage to reproductive organs, we examined the effects of toluene on the male reproductive system in rats, comparing to those of diethylstilbestrol (DES), a potent synthetic estrogen. Toluene (50, 500 mg/kg) or DES (2 mg/kg) injected subcutaneously to male Sprague–Dawley rats once a day for 10 days decreased the epididymal sperm counts and the serum concentrations of testosterone. The mRNA level for gonadotropin-releasing hormone receptor in the pituitary was decreased by DES, but not by toluene. On the contrary, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) formation in testes, the biological marker for oxidative DNA damage, was increased by toluene but not by DES. These results suggest that toluene induces reproductive toxicity via direct oxidative damage of spermatozoa, whereas DES affects endocrine systems via the hypothalamo-pituitary-gonadal axis. Morphological findings supported the idea. To determine the mechanism of 8-oxodG formation *in vivo*, we examined DNA damage induced by toluene metabolic products *in vitro*. Minor toluene metabolites, methylhydroquinone and methylcatechols, induced oxidative DNA damage, and the methylcatechols induced NADH-mediated 8-oxodG formation more efficiently than methylhydroquinone did. We propose that oxidative DNA damage in the testis plays a role in reproductive toxicity induced by toluene.

Keywords: Toluene; Male reproductive toxicity; Oxidative DNA damage; Testis; Diethylstilbestrol; Metabolites

INTRODUCTION

Toluene is widely used as an organic solvent in various industries and commercial products.[1] Toluene may have a severe toxicity on male reproductive organs including epididymal sperm dysfunctions.[1–3] Workers who inhaled toluene-based solvents showed elevated counts of abnormal spermatozoa.[4,5] A long-term experimental carcinogenicity study has shown that toluene is carcinogenic to rats[6] although the International Agency for Research on Cancer (IARC)[7] has assessed that toluene is not classifiable as to its carcinogenicity to humans (group 3). Little is known about the molecular mechanism by which toluene elicits its toxic effects on these male reproductive organs and carcinogenicity.

Diethylstilbestrol (DES) is known as a potent synthetic estrogen, and the inhibitory effects on male reproductive organs by DES can be explained by the same mechanism due to estrogen.[8,9] Estrogen inhibits the testosterone production in Leydig cells in adult rats testes,[10] resulting in dysfunction of spermatogenesis. This inhibition is associated with down regulation of a cytochrome P450 17α-hydroxylase/C17-20-lyase (CYP17) gene expression.[11,12] Gonadotropin-releasing hormone (GnRH) plays a pivotal role in hypothalamo-pituitary-gonadal axis to control the mammalian reproduction.[13] DES

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affects spermatogenesis via hypothalamo-pituitary-gonadal axis.[14] It is reasonable to use DES as the agent which can inhibit male reproductive system via hypothalamo-pituitary-gonadal axis by observing gene expression of GnRH and its receptor in addition to CYP17.

Toluene inhalation altered the hormonal status of the anterior pituitary gland in rodents.[15,16] A recent study has also shown that acute exposure to toluene affects the secretion of gonadotropins in workers.[17,18] These reports suggest that one possible way for the reproductive toxicity of toluene may be related to alterations in the endocrine status. Another possible mechanism of the reproductive toxicity of toluene may involve its direct effects on the reproductive organs. Several reproductive toxicants have DNA damaging effects on germ epithelium of the testis.[19] Our previous study[20] has indicated that minor metabolites of toluene have the ability of oxidative damage to DNA isolated from the human gene, and proposed that toluene may exhibit carcinogenicity and reproductive toxicity via oxidative DNA damage.

To clarify whether the toxicity results from the interference of endocrine systems or direct damage to reproductive organs, we examined the effects of toluene on the male reproductive system in rats, comparing to those of DES. Furthermore, in order to investigate the mechanism of toluene-induced DNA damage *in vivo*, we measured 8-oxodG formation induced by toluene metabolites in calf thymus DNA in *in vitro* system.

MATERIALS AND METHODS

Animals

Seven-weeks-old male Sprague–Dawley rats were purchased from CLEA Japan (Tokyo, Japan). Rats were fed laboratory chow (CE-2, CLEA Japan) and tap water *ad libitum*, and housed with controlled photoperiod (12-h light/12-h dark) and temperature (23 ± 1°C). All procedures for animal experiments were approved by the Experimental Animal Care Committee of Mie University School of Medicine.

Toluene and DES Treatments

Following one week of acclimatization period, rats were divided into four groups: control, low-dose (50 mg/kg body weight) and high-dose toluene (500 mg/kg body weight) treatment, and diethylstilbestrol (DES, 2 mg/kg body weight) treatment. Toluene or DES in olive oil was injected to rats subcutaneously once a day for 10 days. Control animals were injected with the same amount of olive oil. The rats were killed by decapitation after 24 h of the final injection, and the heart, liver, adrenal, testis, epididymis and prostate gland were removed quickly and weighed. All these organs were frozen in liquid nitrogen and stored until use at −80°C, except for right epididymis which was used for counting of spermatozoa. Blood was collected to obtain serum and testosterone was assayed by EIA kit (ICN Pharmaceuticals, Orangeburg, NY). Brains were removed and put onto an ice-cooled glass plate, where hypothalamus and pituitary were dissected according to the method described by Glowinski and Iversen.[21]

Epididymal Sperm Counts

The spermatozoa were determined by the method of Kawashima *et al.*[22] The right cauda epididymis was minced in 5 ml of phosphate buffer (pH 7.2) at 38°C, and the spermatozoa were counted in a hemocytometer.

RT-PCR and Southern Hybridization

Total cellular RNA was isolated from hypothalamus, pituitary and testis using a TRIZOL reagent (Life Technologies, Inc., Grand Island, NY). Reverse transcription-polymerase chain reaction (RT-PCR) were performed by the procedure described previously.[23] The mRNA level for ribosomal protein subunit L19 was measured in each tissue as an internal control. The primers used were: 5′-CACTATGGTCACCAGCGGG-3′ and 5′-AGAGCTCCTCGCACATCCCTAAGA-3′ for GnRH cDNA, 5′-GAAGCCCGTCCTTGGAGAAAT-3′ and 5′-GCGATCCAGGCTAATCACCACCAT-3′ for GnRH receptor cDNA, 5′-CAGGAAAAGTATGGTCCCATCT-3′ and 5′-CCTCCAAGCCTTTGTTGGGAAA-3′ for CYP17 cDNA, and 5′-GAAATCGCCAATGCCAACTC-3′ and 5′-TCTTAGACCTGCGAGCCTCA-3′ for L19 cDNA. The reactions were carried out in a DNA thermal cycler under the following conditions: denaturation at 94°C for 1 min except that the initial denaturation was for 3 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. PCRs were repeated 23 cycles for GnRH, 18 cycles for GnRH receptor, 24 cycles for CYP17, and 14 or 16 cycles for L19. The PCR cycles were confirmed to be within a linear range of quantitative amplification for each RNA. After PCR, the reaction mixtures were electrophoresed on 1.5% agarose gels and blotted onto nylon membranes (Schleicher & Schuell, Inc., Keene, NH). Blotted membranes were hybridized with specific oligonucleotide probes which were labeled with [γ-^{32}P]ATP (ICN Biomedicals Ins., Costa Mesa, CA) using T_4 polynucleotide kinase (Amersham Pharmacia Biotech, Tokyo, Japan). The radioactivities in the bands corresponding to each mRNA were determined by a laser image analyzer

(Fujix BAS1000; Fuji Film, Tokyo, Japan) and were expressed as arbitrary units.

Measurement of 8-oxodG Content in Testis

The testes were minced and gently homogenized in a Dounce homogenizer by 5 strokes in ice-cold phosphate-buffered saline (PBS). The cell suspensions were filtered through a layer of nylon mesh (80 μm) and centrifuged at 1000*g* for 10 min. The pellets were washed twice with PBS. Under anaerobic conditions, DNA was extracted by using RNase A (Sigma Chemical Co., St. Louis, MO) and proteinase K (Boehringer Mannheim, Mannheim, Germany) in a lysis buffer for DNA extraction (Applied Biosystems, Foster City, CA) at 60°C for 1 h. The DNA was enzymatically digested by nuclease P_1 (Yamasa Shoyu Co., Chiba, Japan) and bacterial alkaline phosphatase (Sigma Chemical Co.) to the nucleosides, and analyzed on a HPLC equipped with an electrochemical detector (ECD) as described previously.[24,25]

Morphological Assessment

The testes were dissected out and immersed in fixative solution (2% paraformaldehyde, 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3). Then the testis was cut into small pieces and kept in the same fixative solution for 2 h at room temperature. Subsequently, the tissues were post-fixed with 2% OsO_4 in 0.1 M phosphate buffer (pH 7.3). After alcohol dehydration, the tissues were embedded in Epon 812 (TABB, Berkshire, UK). Semithin sections of 1 μm in thickness were cut on an ultramicrotome MT-5000 (Dupont Sorvall, Newtown, CT) and stained with toluidine blue. The sections were examined with light microscopy.

Analysis of 8-oxodG Formation by Toluene Metabolites in Calf Thymus DNA

DNA fragments from calf thymus were incubated with several toluene metabolites [methylhydroquinone (MHQ, Nacalai Tesque, Inc., Kyoto, Japan) and methylcatechols (MCs; 3-MC and 4-MC, Tokyo Kasei Co., Tokyo, Japan)], $CuCl_2$ and NADH for indicated duration at 37°C. After ethanol precipitation, DNA was enzymatically digested to the nucleosides with nuclease P_1 and calf intestine phosphatase and analyzed by a HPLC-ECD, as described previously.[24]

Statistical Analysis

Data are expressed as means ± SD. Statistical comparison between means was calculated with one way analysis of variance and Student's *t*-test. When the variances were not homogeneous, Mann–Whitney *U*-test was used. Differences were considered significant with $P < 0.05$.

RESULTS

Body and Organ Weights

The effects of toluene and DES treatments on body and organ weights were summarized in Table I. Injections of DES decreased the weights of body and organ except for the liver. The effect of DES was most markedly observed in epididymis and prostate weights. Low-dose toluene treatment had little effect on the body and tissue weights, but high-dose toluene treatment significantly decreased the body, heart and prostate weights.

Effects of Toluene and DES on Sperm Counts and Serum Testosterone Levels

The number of sperm was significantly lowered in high-dose toluene and DES groups than the control group (Fig. 1A). The serum concentrations of testosterone were significantly lowered in the high-dose toluene and DES groups than those in the control group (Fig. 1B). Low-dose toluene tended to reduce the sperm count ($P = 0.0814$) but had no significant differences.

TABLE I Effects of toluene and DES treatments on body and organ weights

	Control	Toluene 50 mg/kg BW	Toluene 500 mg/kg BW	DES 2 mg/kg BW
Body weight (g)	358 ± 8	356 ± 13	335 ± 10*	275 ± 10*
Heart (g)	0.94 ± 0.04	0.87 ± 0.02*	0.87 ± 0.03*	0.75 ± 0.03*
Liver (g)	14.2 ± 0.9	13.7 ± 1.0	13.3 ± 1.2	14.5 ± 1.2
Adrenal (mg)	49 ± 8	53 ± 8	57 ± 13	85 ± 15*
Testis (g)	1.57 ± 0.02	1.62 ± 0.06	1.55 ± 0.12	1.33 ± 0.05*
Epididymis (g)	0.149 ± 0.030	0.154 ± 0.027	0.130 ± 0.033	0.075 ± 0.009*
Prostate gland (g)	0.265 ± 0.040	0.275 ± 0.026	0.203 ± 0.069*	0.071 ± 0.009*

Values are means ± SD for four rats. *$P < 0.05$ *vs.* control.

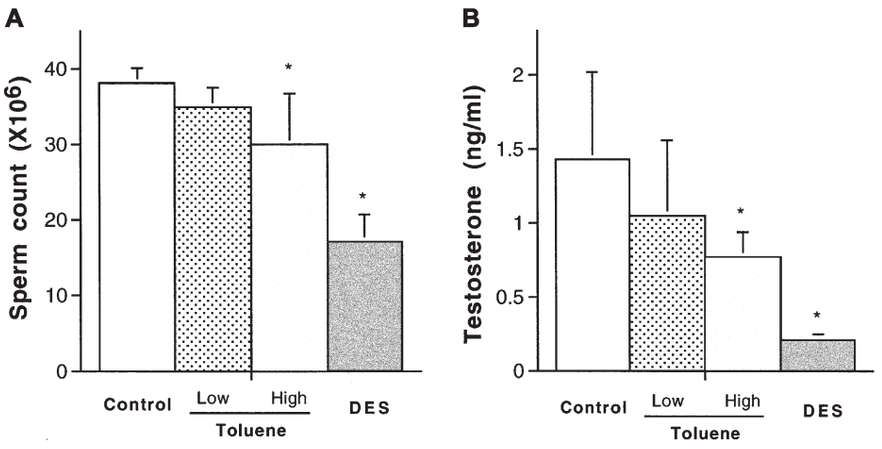

FIGURE 1 Effects of toluene and DES treatments on epididymal sperm counts (A) and concentrations of serum testosterone (B). Low-dose (50 mg/kg body weight) toluene-injected group, High-dose (500 mg/kg body weight) toluene-injected group. Values are means ± SD for four rats. * $P < 0.05$ *vs.* control.

Effects of Toluene and DES on mRNA Expressions for GnRH, GnRH Receptor and CYP17

The mRNA levels for GnRH in hypothalamus tended to lowered in DES group ($P = 0.0591$), although no significant difference was observed (Fig. 2A). To the contrary, administration of DES significantly decreased the mRNA level for GnRH receptor in the pituitary (Fig. 2B). Toluene treatment, however, did not affect the mRNA level for pituitary GnRH receptor (Fig. 2B). The mRNA levels in the testis for CYP17, a key enzyme for testosterone synthesis, was significantly decreased in DES groups (Fig. 2C). Toluene treatment tended to reduce the testis CYP17 mRNA ($P = 0.0814$, 0.1124) but had no significant differences. There was no significant difference in the levels of L19 mRNA, an internal control, among the groups in all tissues tested (data not shown).

Formation of 8-oxodG in Testicular DNA by Toluene

To confirm direct damage to male reproductive organ, the contents of 8-oxodG in DNA extracted from testes were measured. The amount of 8-oxodG was significantly higher in the high-dose toluene group than in the control group (Fig. 3). On the other hand, DES did not increase the amount of 8-oxodG (Fig. 3).

Morphological Findings

The testis in the control group showed regular arrangement of seminiferous epithelium and no degenerating cells in the contortous seminiferous tubules. It was clear that Leydig cells crowded in the interstitial tissue (Fig. 4A). In the high-dose toluene group, moderately degenerating spermatogonia

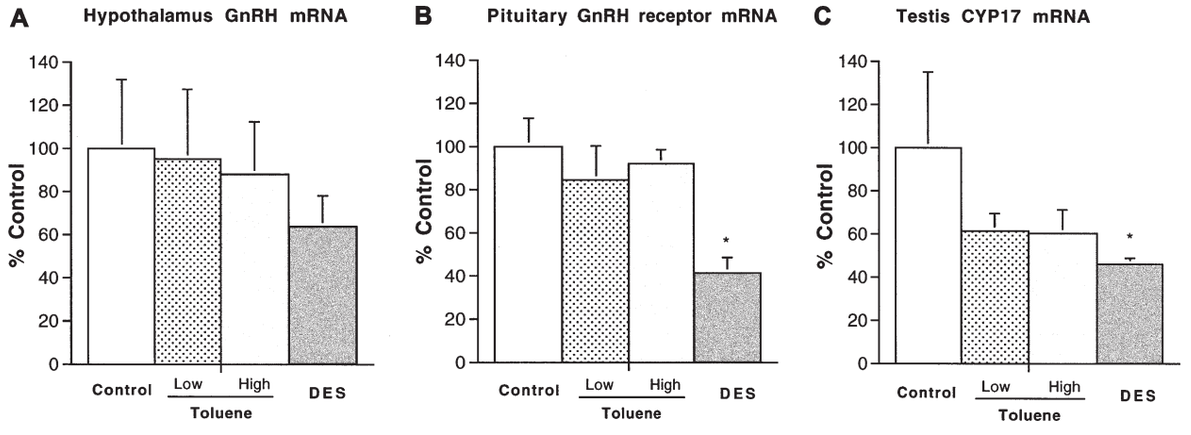

FIGURE 2 Effects of toluene and DES treatments on the GnRH, GnRH receptor and CYP17 mRNA expressions in various tissues. (A) GnRH mRNA in hypothalamus, (B) GnRH receptor mRNA in pituitary, (C) CYP17 mRNA in testis. Low-dose (50 mg/kg body weight) toluene-injected group, High-dose (500 mg/kg body weight) toluene-injected group. Values are means ± SD for four rats. * $P < 0.05$ *vs.* control.

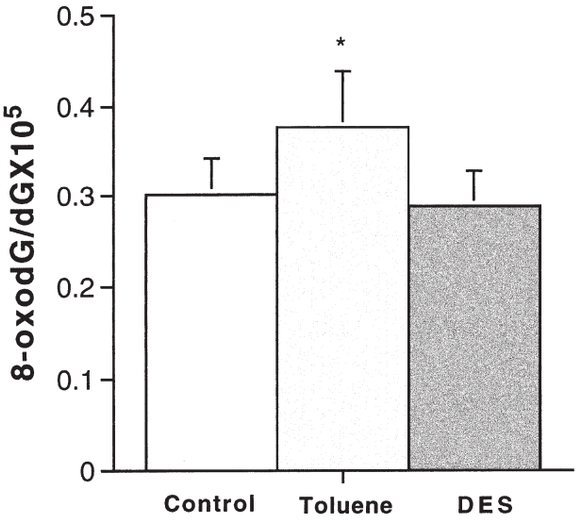

FIGURE 3 Effects of toluene and DES treatments on the formation of 8-oxodG in the testis. High-dose (500 mg/kg body weight) toluene-injected group. Values are means ± SD for five to six rats. * $P < 0.05$ *vs.* control.

were observed in the testis. Moderate numbers of spermatogonia, located in the basement of seminiferous epithelium, showed cytoplasmic vacuolization. On the contrary, the Leydig cells were preserved almost well in the interstitial tissues, but Leydig cells showed a little atrophic appearance (Fig. 4B). In the DES group, it was prominent that severe degenerating spermatogonia were observed in the testis. Many spermatogonia showed severe cytoplasmic vacuolization and some of them were fell away. Markedly decreased numbers of Leydig cells were scattered in the interstitial tissue of testes in them. It was also prominent that remained Leydig cells were atrophic (Fig. 4C).

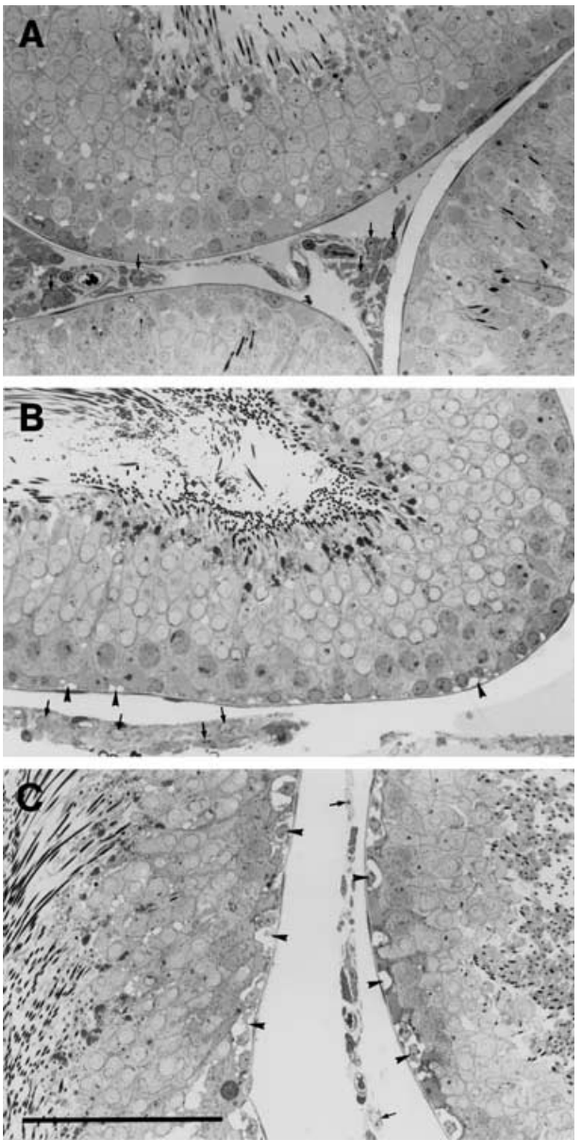

FIGURE 4 Effects of toluene and DES treatment on the morphological findings in the testis. Testis in the control group (A). Testis in the high-dose toluene-injected group (B). Testis in the DES-injected group (C). A, B and C are same magnification. Scale bar = 100 μm. Arrows indicate Leydig cells in the interstitial tissue. Arrow heads indicate spermatogonia with cytoplasmic vacuolization located in the basement of seminiferous epithelium.

Formation of 8-oxodG in Calf Thymus DNA by Toluene Metabolites

We measured 8-oxodG contents in calf thymus DNA treated with toluene metabolites, MHQ and MCs (3-MC and 4-MC) in the presence and absence of Cu(II) and NADH (Fig. 5). The metabolites significantly induced Cu(II)-dependent 8-oxodG formation, and the potency was similar. However, MCs-induced 8-oxodG formation was much higher than that of MHQ in the presence of NADH. These metabolites did not induce the increase of 8-oxodG formation in the absence of Cu(II).

DISCUSSION

The present study have revealed that the mechanism of toluene-induced male reproductive toxicity is different from that of DES. The most remarkable finding of this study is that the toluene treatment increased 8-oxodG formation in the testis, while the DES treatment did not. On the other hand, DES decreased the gene expression of GnRH receptor in the pituitary but toluene did not. These results imply toluene-induced reproductive toxicity is attributed to direct damage of testis while DES affects endocrine systems via the hypothalamo-pituitary-gonadal axis. The idea was supported by morphological findings. That is, toluene mainly damaged

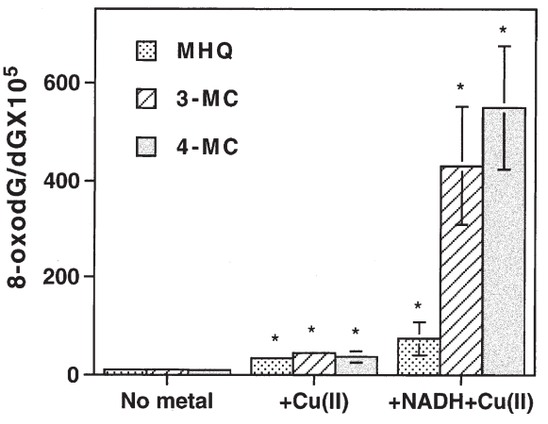

FIGURE 5 Formation of 8-oxodG in calf thymus DNA by toluene metabolites (MHQ, 3-MC and 4-MC) in the presence and absence of NADH and Cu(II). Calf thymus DNA fragments (100 μM per base) were incubated with 5 μM metabolite, 200 μM NADH and 20 μM $CuCl_2$ for 60 min at 37°C. After ethanol precipitation, DNA was enzymatically digested to the nucleosides and analyzed by HPLC-ECD. Values are means ± SD of three to four independent experiments. * $P < 0.05$ *vs.* control (no metal conditions).

spermatogonia while DES strongly damaged both Leydig cells and spermatogonia. Low serum level of testosterone was also observed in toluene-treated group. The decrease in weight of heart and prostate gland by the treatment of toluene may relate to low serum level of testosterone, since castration decreases prostate weight[26] and heart weight.[27] Testosterone is catalyzed by CYP17 which is highly expressed in Leydig cells. This may be interpreted as dysfunction of Leydig cells which are a little damaged. It is noteworthy that toluene-induced oxidative DNA damage of testis including 8-oxodG formation resulted in male reproductive toxicity. Relevantly, 8-oxodG levels in human sperm are closely associated with the impairment of sperm function and with male infertility.[28]

To determine the mechanism of 8-oxodG formation *in vivo*, we examined DNA damage induced by toluene metabolites *in vitro*. Metabolic studies have revealed that toluene is mainly oxidized at methyl group, a series of oxidations leads to benzoic acid, which is detoxicantly conjugated with glycine to form hippuric acid, which is then rapidly excreted in urine. Cresol is a minor metabolite of toluene, and a part of it is further metabolized to MHQ and MCs by aromatic hydroxylation.[29] We reported that MHQ caused oxidative DNA damage but benzoic acid and hippuric acid did not by using the experiments *in vitro*.[20] Furthermore, Shen showed that MCs were the most cytotoxic metabolites of benzene, toluene, ethylbenzene and xylene.[30] In the present study, we found that MCs induced oxidative DNA damage more efficiently than MHQ in the presence of endogenous reductant NADH. Therefore, we speculated that these metabolites might participate in the male reproductive toxicity via oxidative DNA damage. If metabolizing enzymes would act more efficiently in aromatic hydroxylation than methyl hydroxylation, toluene might have stronger reproductive toxicity and carcinogenicity. Possible mechanisms of oxidative DNA damage by the metabolites of toluene can be envisioned as follows. Dihydroxy forms of toluene metabolites such as MHC and MCs, are autoxidized to semiquinone radicals, and further to benzoquinone forms.[20] Generation of $O_2^{\bullet -}$ occurs coupled with the autoxidation of the metabolites. Thereafter, $O_2^{\bullet -}$ is dismutated to generate H_2O_2. In the presence of metal ions, H_2O_2 causes oxidative DNA damage. The addition of NADH enhanced oxidative DNA damage by MCs more efficiently than that by MHQ. It is revealed that catechol and hydroquinone themselves have different redox properties leading to different DNA-damaging ability.[31,32] The oxidized form of catechol, 1,2-benzoquinone is converted directly into catechol through two-electron reduction by NADH, whereas the oxidized form of hydroquinone, 1,4-benzoquinone into semiquinone radical through one-electron reduction.[31,32] This can also explain the different NADH-mediated DNA-damaging ability between MCs and MHQ. Therefore, it is a reasonable idea that these metabolites may participate in the male reproductive toxicity of toluene by acting directly on testis via oxidative stress. Relevantly, Mattia *et al.* have shown that toluene or its metabolites can stimulate the formation of reactive oxygen species inducing lipid peroxidation in various organs.[33,34] The motility of rabbit spermatozoa was decreased by lipid peroxidation,[35] and *in vitro* exposure to toluene impaired sperm motility.[36] These reports support the idea that oxidative DNA damage induced by toluene is involved in its male reproductive toxicity.

Carcinogenic benzene[37] and nitrobenzene[38] are reproductive toxicants as well as toluene, and their metabolites are also known to cause oxidative DNA damage.[39–41] Recently, we reported that trinitrotoluene (TNT) induced reproductive toxicity via oxidative DNA damage by its metabolite.[42] Genotoxic agents can reduce sperm counts, resulting in infertility.[43,44] Rapidly dividing cells are sensitive to genotoxic insults because of their low activity of DNA repair enzymes.[45] Dalgaad *et al.* reported that pre- and postnatal exposure to toluene resulted in apoptotic neurogeneration but no effect on semen quality in weaned male rats.[46] The rapidly dividing cells such as neurons in peri-natal period and spermatogonia cells in adulthood may be vulnerable to toluene. The male reproductive organs are highly susceptible to oxidative damage,[28,47–49] since testes have low activities of catalase,[47,50] and testicular cells such as spermatocytes and Sertoli cells showed little or no activity of Cu, Zn-SOD.[51] In this study,

we demonstrated that toluene administration decreased sperm number and increased 8-oxodG formation in sperm cells of the testis. This oxidative DNA damage *in vivo* may be attributed to the damage induced by toluene metabolites as shown by the experiments *in vitro*. In conclusion, toluene has direct toxic effects on male reproductive organs acting on their DNA rather than disrupting the hypothalamic-pituitary-testis axis. This is the first report to demonstrate that reproductive toxicity of toluene may be associated with oxidative DNA damages in the testis.

Acknowledgements

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