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2,4,6-Trinitrotoluene-induced Reproductive Toxicity via Oxidative DNA Damage by its Metabolite

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Several epidemiological studies and animal experiments showed that 2,4,6-trinitrotoluene (TNT), a commonly used explosive, induced reproductive toxicity. To clarify whether the toxicity results from the interference of endocrine systems or direct damage to reproductive organs, we examined the effects of TNT on the male reproductive system in Fischer 344 rats. TNT administration induced germ cell degeneration, the disappearance of spermatozoa in seminiferous tubules, and a dramatic decrease in the sperm number in both the testis and epididymis. TNT increased the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in sperm whereas plasma testosterone levels did not decrease. These results suggest that TNT-induced toxicity is derived from direct damage to spermatozoa rather than testosterone-dependent mechanisms. To determine the mechanism of 8-oxodG formation *in vivo*, we examined DNA damage induced by TNT and its metabolic products *in vitro*. 4-Hydroxylamino-2,6-dinitrotoluene, a TNT metabolite, induced Cu(II)-mediated damage to ^{32}P-labeled DNA fragments and increased 8-oxodG formation in calf thymus DNA, although TNT itself did not. DNA damage was enhanced by NADH, suggesting that NADH-mediated redox reactions involving TNT metabolites enhanced toxicity. Catalase and bathocuproine inhibited DNA damage, indicating the involvement of H_2O_2 and Cu(I). These findings suggest that TNT induces reproductive toxicity through oxidative DNA damage mediated by its metabolite. We propose that oxidative DNA damage in the testis plays a role in reproductive toxicity induced by TNT and other nitroaromatic compounds.

Keywords: 2,4,6-Trinitrotoluene; Reproductive toxicity; DNA damage; Reactive oxygen species; Testis; Epididymis

Abbreviations: TNT, 2,4,6-trinitrotoluene; HADNT, 4-hydroxylamino-2,6-dinitrotoluene; ADNT, 4-amino-2,6-dinitrotoluene; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; DTPA, diethylenetriamine-*N,N,N′,N″,N‴*-pentaacetic acid; DMSO, dimethylsulfoxide; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatograph(y); HPLC–ECD, electrochemical detector coupled to HPLC; ANOVA, analysis of variance; ·OH, free hydroxyl radical; O_2^-, superoxide anion radical; HOMO, highest occupied molecular orbital

INTRODUCTION

2,4,6-Trinitrotoluene (TNT) is used to produce explosives, used in munitions and mining. Recently, remediation of soils and water contaminated with TNT is of concern.[1–3] Chinese male workers exposed to TNT complained sexual disorders such as loss of libido and impotence.[4] The volume of semen and percentage of motile spermatozoa were found to decrease, and the sperm malformation incidence increased in TNT-exposed workers.[5] Animal experiments revealed that TNT caused diminution of spermatozoa due to testicular atrophy observed by histological examination.[6,7] The

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mechanisms, however, whereby TNT induces reproductive toxicity have not been characterized.

Reproductive toxicants exert toxicity through effects on the endocrine system and/or direct damage to reproductive organs. Genotoxic agents are thought to directly damage reproductive organs via DNA damage. The germline epithelium of the testis, a highly proliferative tissue, is susceptible to the DNA-damaging effects of environmental toxicants.[8] The DNA repair activity of enzymes in spermiogenic cells is low; thus, these cells are highly sensitive to genotoxic insult.[9] TNT is metabolized to 4-hydroxylamino-2,6-dinitrotoluene (HADNT) and 4-amino-2,6-dinitrotoluene (ADNT).[10,11] Thus, TNT metabolites may be involved in reproductive toxicity.

In the present study, we characterized the effects of TNT on male reproductive organs in Fischer 344 rats. We examined the pathological alterations and TNT accumulation in the rats. To define the mechanism of reproductive damage, we measured the plasma testosterone level to detect the disturbance of endocrine system, and the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in rat epididymides and testes, as an indicator of oxidative DNA damage.[12–14] To investigate the mechanism of TNT-induced DNA damage, we examined the oxidative damage induced by TNT and its metabolites to ^{32}P-5′-end-labeled DNA fragments, obtained from human genes, and measured the formation of 8-oxodG in calf thymus DNA.

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MATERIALS AND METHODS

Materials

TNT, HADNT and ADNT [purity of all reagents ≧ 95%] were synthesized as described by McGookin *et al.*[15] Restriction enzymes (ApaI, AvaI, EcoRI, PstI and MroI) and T_4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA, USA). Calf intestine phosphatase was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). [γ-^{32}P]ATP (222 TBq/mmol) was purchased from New England Nuclear (Boston, MA, USA). Diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were obtained from Dojin Chemicals (Kumamoto, Japan). NADH was purchased from Kohjin (Tokyo, Japan). Acrylamide, dimethylsulfoxide (DMSO), bisacrylamide, piperidine and hydrogen peroxide (H_2O_2) were obtained from Wako Chemicals (Osaka, Japan). $CuCl_2$, ethanol and D-mannitol were procured from Nacalai Tesque (Kyoto, Japan). Calf thymus DNA, superoxide dismutase (SOD, 3000 units/mg from bovine erythrocytes), methional and catalase (45,000 units/mg from bovine liver) were purchased from Sigma Chemical (St. Louis, MO, USA). Nuclease P_1 was obtained from Yamasa Shoyu (Chiba, Japan).

Animals and Treatments

Experiment 1: Male Fischer 344 rats (6 weeks, 3–5 rats per group) were obtained from Clea Japan (Tokyo, Japan). Animals were allowed to acclimatize in an environment with a controlled temperature, humidity and light/dark cycle for 1 week before the experiments. All animal procedures received prior approval from the University Laboratory Animal Care and Use Committee and met current local regulations. TNT was suspended in corn oil and administered to rats by oral injection at a dose of 300 mg/kg (six times per week for 2 weeks). For controls, rats given corn oil (2 ml/kg) were used. Body weight and testicular length were determined at the time just before injection of TNT (at approximately 8 a.m.). One hour and 2 weeks after the last TNT administration, the rats were killed by withdrawing approximately 7 ml of blood from heart under ether anesthesia. After removal, organs were weighed.

Experiment 2: TNT, suspended in corn oil, was administered to Male Fischer 344 rats (10 weeks, 4–8 rats per group) by oral injection at a dose of 300 mg/kg/day for 2 days. As a control, rats were used to receive corn oil (2 ml/kg) only. Two hours after the last TNT administration, rats were killed as described above. Testes and epididymides were removed and kept in ice-cold phosphate-buffered saline (PBS). Immediately, they were subjected to measurement of 8-oxodG formation.

Histopathology

Tissue specimens (4 μm) were stained with Carrazzi's hematoxylin and the periodic acid Schiff (PAS) reaction, as described.[16]

Sperm Head Counts

Testicular and epididymal sperm counts were determined as described by Amann *et al.*[17] with the following modifications. Briefly, the testes and cauda epididymides were homogenized using Polytron in 75 ml of saline with 0.05% Triton X-100 (Kinematica, Luzern, Switzerland) for 90 min at speed setting 3. The homogenate was then mixed with an equal volume of 0.2% trypan blue; sperm heads were counted using a hemocytometer.

Assay of Testosterone

Testosterone was extracted from blood plasma samples using an *n*-hexane-diethylether mixture (3:2, v/v). The testosterone concentration in plasma

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was determined using a radioimmunoassay kit (Eiken Kagaku, Tokyo, Japan).

Determination of TNT and its Metabolites in Tissues by HPLC

Tissues were homogenized in four volumes of 1.15% KCl. Following the mixture of these homogenates (0.4–1 ml) with 10 volumes of benzene, TNT and its metabolites were extracted. After centrifugation at 3000*g* for 10 min, benzene layers were evaporated to dryness under nitrogen gas. The remaining residue was resuspended in 0.2 ml DMSO, and then filtered through a 0.5 μm membrane filter (Millipore, Tokyo, Japan). Each filtrate was examined by high performance liquid chromatography (HPLC). The chromatographic equipment comprised an LC-10AT pump, an SPD-10A UV–Vis detector from Shimadzu (Kyoto, Japan) and a chromatocorder 11 integrator from System Instruments (Tokyo, Japan). HPLC analysis with UV absorbance detection at 255 nm was performed using a YMC AM (ODS) column (250 × 4.6 mm I.D., Yamamura Chemical Labs., Kyoto, Japan) at a flow rate of 1 ml/min. The mobile phase consisted of water and acetonitrile (1:1, v/v). Quantification was performed utilizing *m*-nitroaniline as an internal standard. Under these conditions, the retention times of TNT and *m*-nitroaniline were 12.5 and 6.2 min, respectively.

Measurement of 8-oxodG Formation in Rat Testes and Epididymides

Testes and caput epididymides were gently homogenized using a Dounce homogenizer (5 strokes) in 10 volumes of ice cold PBS (pH 7.4), and then filtered through a nylon mesh (144-μm pore size) as previously described.[18] The homogenates were then centrifuged at 1000*g* for 5 min; the pellets were stored at −70°C prior to DNA extraction. DNA was extracted from the filtered homogenate by using a DNA Extractor WB Kit (Wako, Osaka, Japan); these homogenates were then digested into nucleosides with nuclease P_1 and bacterial alkaline phosphatase. The quantity of 8-oxodG was measured utilizing an electrochemical detector coupled to HPLC (HPLC–ECD) as described by Kasai *et al.*[19]

Measurement of 8-oxodG Formation in Calf Thymus DNA

Calf thymus DNA (100 μM/base) was incubated with the indicated concentrations of TNT, HADNT or ADNT in 4 mM sodium phosphate buffer (pH 7.8) containing 100 μM NADH and 20 μM $CuCl_2$ at 37°C for 60 min. 0.2 mM DTPA was added to stop the reaction. Following ethanol precipitation, DNA was digested into nucleosides by incubation with nuclease P_1 and alkaline phosphatase and analyzed by HPLC–ECD as previously described.[20]

Preparation of ^{32}P-5′-end-labeled DNA Fragments

DNA fragments were obtained from the human c-Ha-*ras*-1 protooncogene[21] and the tumor suppressor genes, *p*16[22] and *p*53.[23] The DNA fragment containing exon 1 of the *p*16 tumor suppressor gene was obtained by PCR amplification of human genomic DNA, using 5′ and 3′ amplimers synthesized by Funakoshi Co. The PCR product was ligated into pGEM®-T Easy Vector (Promega Corporation) and transferred into Epicurian coli® supercompetent cells (Stratagene). This vector was digested with EcoRI; the resulting DNA fragment was fractionated by electrophoresis on a 2% agarose gel. Dephosphorylation with calf intestine phosphatase followed by phosphorylation with [γ-^{32}P]ATP in the presence of T_4 polynucleotide kinase yielded a 5′ end-labeled 490-base pair fragment (*Eco*RI* 5841-*Eco*RI* 6330) containing exon 1. The 490-base pair fragment was further digested with MroI to obtain a singly labeled 328-base pair fragment (*Eco*RI* 5841-*Mro*I 6168). The DNA fragment of the *p*53 tumor suppressor gene was prepared from a pUC18 plasmid. The ^{32}P-5′-end-labeled 443-base pair fragment (*Apa* I 14179-*Eco*RI* 14621) was obtained as previously described.[24] Additional DNA fragments were prepared from the PbcNI plasmid, carrying a 6.6-kb BamHI chromosomal DNA segment containing the human c-Ha-*ras*-1 protooncogene.[25,26] The singly-labeled 98-base pair fragment (*Ava*I* 2247-*Pst* I 2344) was obtained as previously described.[25,26] Asterisks indicate ^{32}P-labeling.

Detection of Damage to Isolated DNA

The standard reaction mixture contained 100 μM NADH, 20 μM $CuCl_2$, ^{32}P-5′-end-labeled DNA fragment, 10 μM calf thymus DNA, and either TNT, HADNT or ADNT in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA in a 1.5 ml microtube. Following a 60 min incubation at 37°C, DNA fragments were heated at 90°C in 1 M piperidine for 20 min and treated as described previously.[25] Experiments were performed in air-saturated solution.

Preferred cleavage sites were determined by a direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam-Gilbert procedure[27] using a DNA-sequencing system (LKB 2010 Macrophor). A laser densitometer (LKB 2222 UltroScan XL) was utilized to measure the relative amounts of oligonucleotides derived from the treated DNA fragments.

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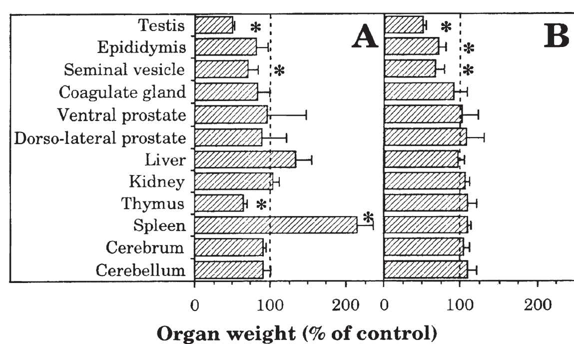

FIGURE 1 Effects of TNT exposure on the weights of rat organs. TNT was administered to rats by oral injection at a dose of 300 mg/kg/day for 2 weeks (six times per week). This figure shows the weights of rat organs at 1 h (A) and 2 weeks (B) after the final injection. Values are expressed as a percent of the control, obtained from each experimental period. Each point is the mean ± SD of 3–5 animals. *, Significantly different from the controls, $p < 0.05$.

Statistics

Data are expressed as means ± standard deviations for each group. When statistically significant F values were obtained ($p < 0.05$), the analysis of variance (ANOVA) was adjusted for Bonferroni's correction factor.

RESULTS

Effects of TNT Administration on Male Reproductive Organs

TNT treatment at a dose of 300 mg/kg (six times per week for 2 weeks, oral injection) did not result in clinical signs of toxicity or decreases in body weight (data not shown). Testicular length, however, was diminished by 17% at 13 days after the first injection. Completion of the TNT-administration resulted in a 7–20% decrease in testicular length. TNT also altered the organ weights of treated rats (Fig. 1). The weights of the testis and seminal vesicles decreased to 49 and 71% of the control, respectively, measured 1 h after the final TNT injection. Two weeks after the final injection, these decreases in organ weights continued. The epididymis demonstrated weight loss 2 weeks after the final injection (69% of the control); the weight of ventral- and dorsolateral-lobes of the prostate and coagulate gland, however, did not decrease. Overall, animals exhibited a marked atrophy of the testis and epididymis induced by TNT (Fig. 2). In addition, we observed a significant loss in thymic weight and an increase in splenic weight 1 h after the final injection. The weight of the liver increased to 134% of the control (but not significant). After 2 weeks, the liver, thymus, and spleen weights returned to control levels. We did not observe an effect of TNT on the organ weight of the kidney, cerebrum, and cerebellum. These results suggest that TNT is toxic specifically to reproductive organs.

Morphological Changes in Testis and Epididymis Induced by TNT Administration

TNT also induced histological alterations in the rat testis and epididymis (Fig. 3). The testis of TNT-treated rats (Fig. 3B–D) exhibited severe histological alterations compared to controls (Fig. 3A). No spermatozoa were observed in the seminiferous tubules at 1 h after the final TNT-injection. Several multinucleated giant cells and condensed nuclei could also be identified. The lack of multiple steps in germ cell development prevented us from distinguishing complete stage profiles associated with

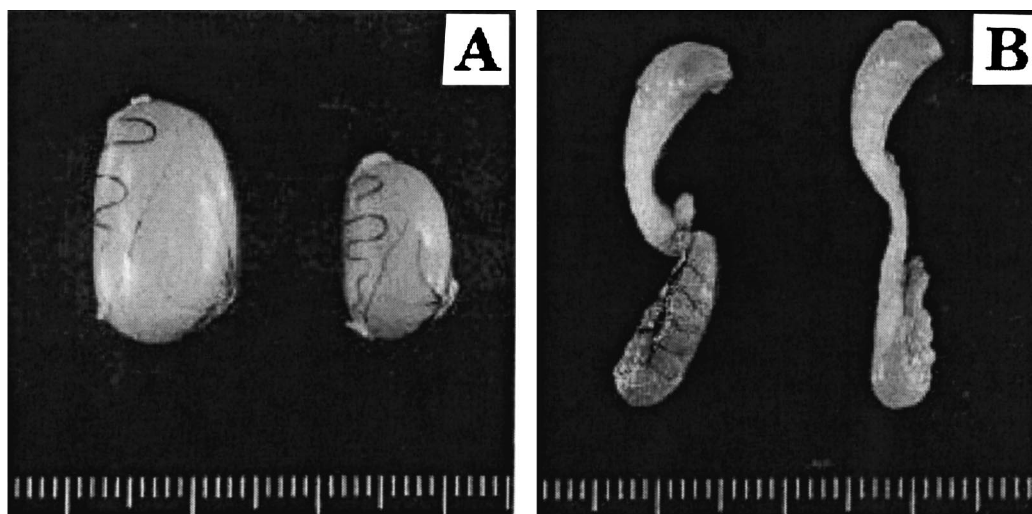

FIGURE 2 TNT-induced atrophy of rat testis and epididymis. Each photo displays the testis (A) and epididymis (B) from the control rats on the left and the corresponding organs from TNT-treated rats at 2 weeks after the final injection on the right. Subacute TNT exposure resulted in diminution in size of both testis and epididymis.

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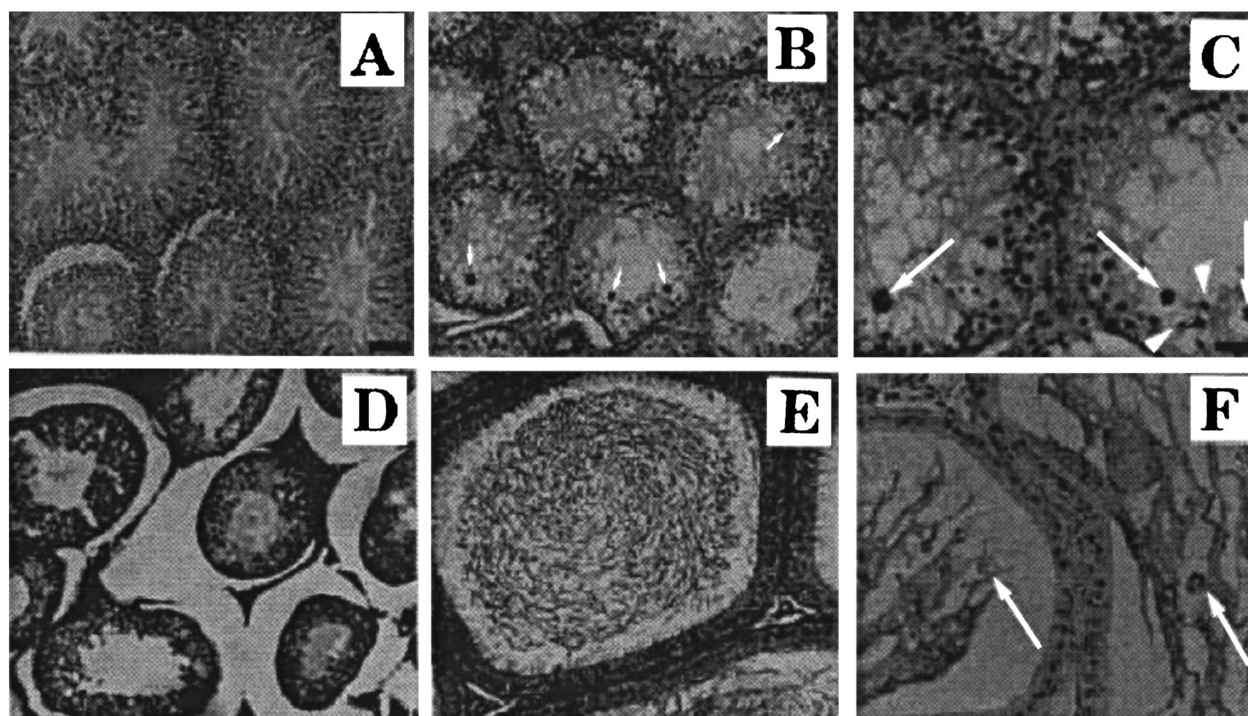

FIGURE 3 Light microscopy of testicular (A–D) and epididymal (E and F) sections. (A) and (E), control; (B), (C), and (F), at 1 h after the final injection of TNT (300 mg/kg/day, six times per week for 2 weeks); (D), 2 weeks after the final injection. Seminiferous tubules contain both multinucleated giant cells (arrows) and condensed nuclei (arrow heads), but do not contain spermatozoa after the subacute TNT exposure (B and C). Two weeks after completion of TNT administration, the tubular lumen was still devoid of spermatozoa (D). In the lumen of epididymal tubules, multinucleated giant cells sloughed-off from seminiferous tubules are observed instead of spermatozoa (F). Bar, 100 μm.

the seminiferous epithelial cycle. In contrast, we observed minimal alteration in Leydig cells, localized to the interstitium. Two weeks after completion of TNT administration, the tubular lumen was still devoid of spermatozoa (Fig. 3D). The epididymal tissue from animals treated with TNT (Fig. 3F) was also altered from controls (Fig. 3E). Few spermatozoa were present in the lumen. Sloughed-off multinucleated giant cells from seminiferous tubules and residual fluid were also observed. The epithelium was found to have degenerated in some places.

Testicular and Epididymal Sperm Number

We performed further analysis of sperm count for the whole testis and cauda epididymis (Table I). Two weeks after the final injection of TNT, sperm numbers in both the testis and epididymis were reduced to 10 and 7% of control levels, respectively. We also observed testicular sperm abnormalities, such as broken heads.

TABLE I Effects of TNT exposure on sperm number in the testis and cauda epididymis

Sperm number (10^6 sperms/organ)	Control	TNT
Testis	135 ± 13	13 ± 2*
Cauda epididymis	26 ± 17	1.8 ± 0.4*

Sperm counts were obtained from testicular and epididymal specimens obtained 2 weeks after the final injection of TNT. Values are mean ± SD of 4–5 animals. *, Significantly different from control, $p < 0.05$.

Plasma Testosterone Concentration

TNT exposure did not decrease plasma testosterone concentrations (Fig. 4). Two weeks after completing TNT administration, the plasma testosterone levels in TNT-treated rats were significantly increased.

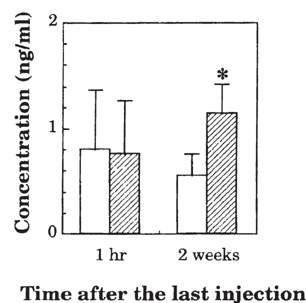

FIGURE 4 Effects of TNT exposure on plasma testosterone concentrations. (A) Levels at 1 h after the final injection (300 mg/kg/day, six times per weeks for 2 weeks) and (B) 2 weeks after the final injection. Each point is the mean ± SD of 3–5 animals. Open column, control rats; stripe column, TNT-treated rats. *, Significantly different from control, $p < 0.05$.

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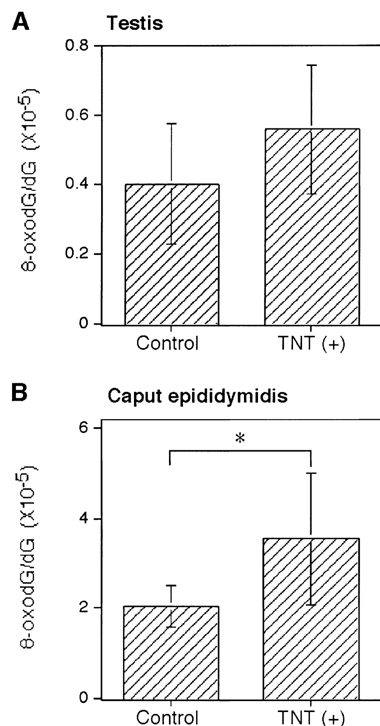

FIGURE 5 8-oxodG formation in rat testis (A) and epididymis (B). TNT was administered to rats by oral injection at a dose of 300 mg/kg/day for 2 days. Each point is the mean ± SD of 4–8 animals. *, Significantly different from control ($p < 0.05$).

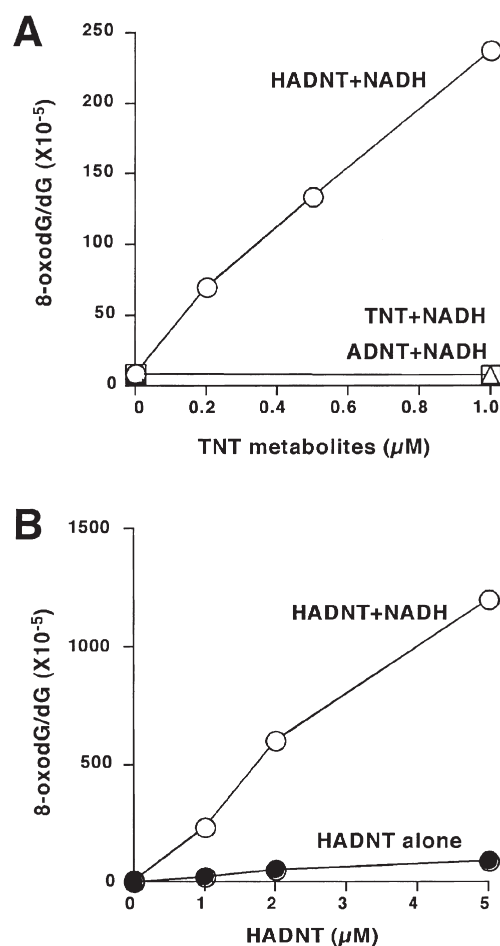

FIGURE 6 8-oxodG formation induced by TNT and its metabolites. (A) 8-oxodG formation by TNT, HADNT or ADNT in the presence of NADH and Cu(II). The reaction mixture contained calf thymus DNA (100 μM/base), 20 μM $CuCl_2$ and the indicated concentrations of TNT (△), HADNT (○), or ADNT (□) in 400 μl of 4 mM sodium phosphate buffer (pH 7.8), containing 2.5 μM DTPA. (B) Effect of NADH on 8-oxodG formation. The reaction mixture contained calf thymus DNA (100 μM/base), 20μM $CuCl_2$, the indicated concentrations of HADNT and no (●) or 100 μM (○) NADH in 400 μl of 4 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. After a 60 min incubation at 37°C, 0.1 mM DTPA was added to stop the reaction. The DNA was then precipitated in ethanol. The DNA fragments were enzymatically digested into nucleosides. 8-oxodG formation was then measured using HPLC–ECD.

Accumulation of TNT in the Testis

The TNT levels within tissues were determined in the testis, liver, kidney, thymus, and spleen. The TNT concentration in the testis was 3.29 ± 1.29 nmol/g at 1 h following the final TNT injection. The accumulation of TNT within the testis was maintained for 2 weeks after the final injection, at 56% of the level (1.84 ± 0.49 nmol/g) at 1 h after the final injection. In contrast, 1 h after the final injection, we could not detect TNT in the liver, 0.3–3.2-fold of the levels found in the testes accumulated in the kidney, thymus, and spleen. Two weeks later, however, TNT was no longer detectable in these organs. Therefore, TNT has a tendency to accumulate in the testis. ADNT levels in testis were also measured. The ADNT concentration was 8.88 ± 1.02 nmol/g at 1 h and 0.70 ± 0.31 nmol/g (8% of the level at 1 h) at 2 weeks following the final TNT injection. These results suggest that HADNT was formed in the testis.

8-oxodG Formation in the Testis and Epididymis after TNT Exposure

TNT induced 8-oxodG formation in the rat testis and caput epididymis (Fig. 5). TNT administration increased 8-oxodG formation in the testes, although the difference was not statistically significant (Fig. 5A). TNT significantly increased the formation of 8-oxodG in sperm cells, obtained from the caput epididymis ($p < 0.05$) (Fig. 5B). In the control groups, the values in the caput epididymis (2.03 ± 0.46) were approximately 5-fold higher than those in the testes (0.40 ± 0.17). These results are consistent with a previous study that 8-oxodG formation significantly increased only in the epididymis in iron-treated rats, and that in the control groups, 8-oxodG content in epididymal sperm cells was higher than that in the testes.[28] Sperm cells have low activity of DNA repair enzymes.[9] Therefore, it is suggested that epididymal sperm cells appear to be more susceptible to oxidative stress compared with testicular cells.

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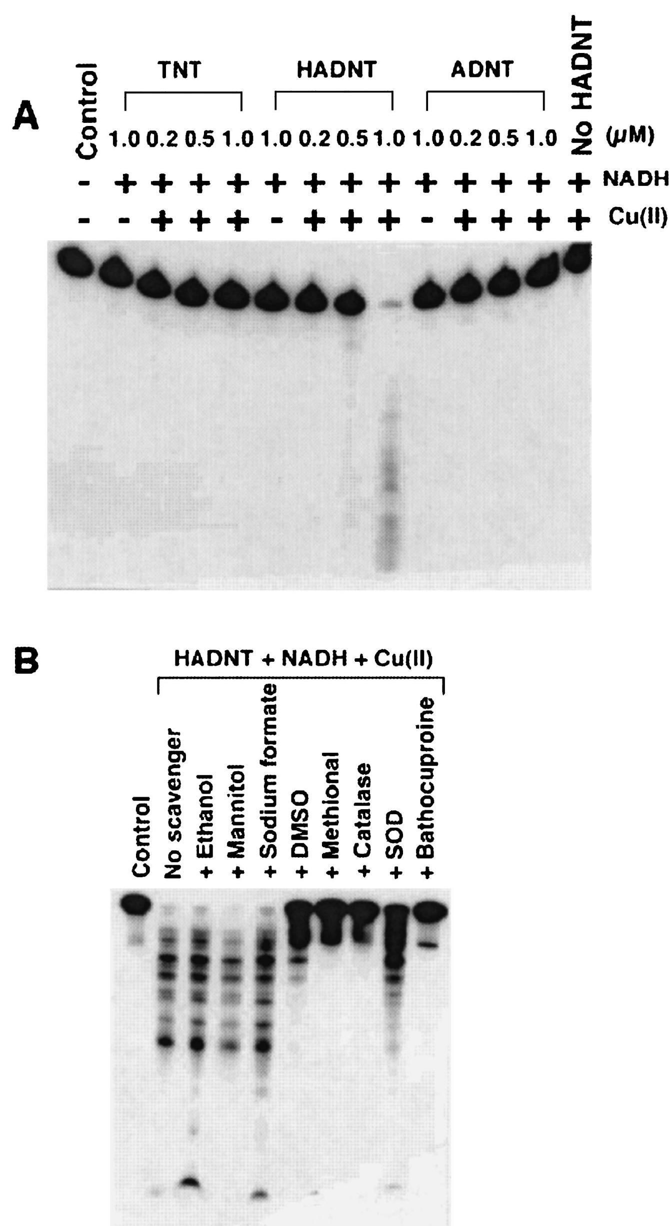

FIGURE 7 DNA damage by TNT, HADNT or ADNT in the presence of Cu(II). The reaction mixture contained the ^{32}P-5′-end-labeled 98-base pair (A) or 443-base pair DNA fragment (B), 10 μM/base of calf thymus DNA, 100 μM NADH and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA with the indicated concentrations of TNT, HADNT or ADNT. These mixtures were incubated for 60 min at 37°C. The DNA fragments were then treated with 1 M piperidine for 20 min at 90°C and electrophoresed on an 8% polyacrylamide/8 M urea gel. Autoradiograms were obtained following exposure of the gel to X-ray film. (B) Where indicated, 10% (v/v) ethanol, 0.1 M mannitol, 0.1 M sodium formate, 10% (v/v) DMSO, 0.1 M methional, 30 units of catalase, 30 units of SOD or 50 μM bathocuproine was added. Controls did not contain HADNT, NADH or Cu(II).

8-oxodG Formation in Calf Thymus DNA Induced by TNT and its Metabolites

Figure 6A shows 8-oxodG formation in calf thymus DNA treated with TNT and its metabolites, HADNT and ADNT, in the presence of NADH and Cu(II). HADNT increased the 8-oxodG formation in a dose-dependent manner, whereas TNT and ADNT did not. NADH dramatically enhanced the 8-oxodG formation by HADNT in the presence of Cu(II) (Fig. 6B).

DNA Damage by TNT and its Metabolites in the Presence of NADH and Metal Ions

Autoradiograms of DNA damage induced by TNT, HADNT or ADNT demonstrated that HADNT induced DNA damage in a dose- (Fig. 7A) and time-dependent manner (data not shown), in the presence of NADH and Cu(II). TNT and ADNT, however, did not (Fig. 7A). NADH enhanced Cu(II)-mediated DNA damage by HADNT (data not shown). DNA damage was enhanced by piperidine

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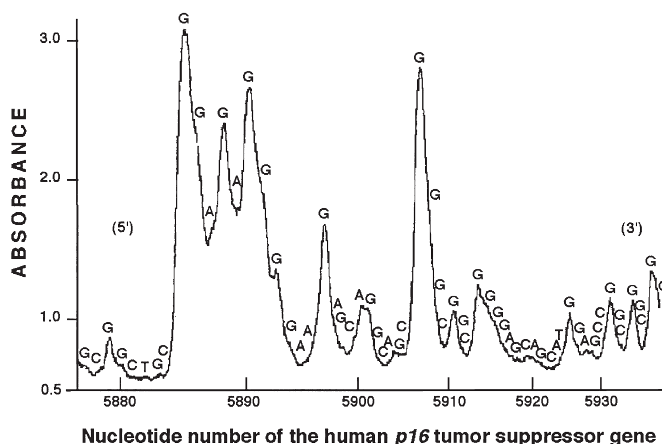

FIGURE 8 Site specificity of DNA cleavage induced by HADNT in the presence of NADH and Cu(II). The reaction mixture contained either the ^{32}P-5′-end-labeled 328-base pair DNA fragment, 10 μM/base of calf thymus DNA, 1 μM HADNT, 100 μM NADH and 50 μM $CuCl_2$ in 10 mM sodium bicarbonate buffer (pH 7.8), containing 2.5 μM DTPA. After a 60 min incubation at 37°C, DNA fragments were treated as described in Fig. 7. The relative amounts of DNA fragments were measured by laser scanning densitometry (LKB 2222 UltroScan XL). The horizontal axis shows the nucleotide number of the human *p*16 tumor suppressor gene.

treatment (data not shown), suggesting that HADNT causes both backbone breakage and base modification. HADNT plus NADH did not cause DNA damage in the absence of metal ion (Fig. 7A) or in the presence of Fe(II), Fe(III) or Mn(II) (data not shown). The addition of catalase and bathocuproine, a Cu(I)-specific chelator, inhibited the DNA damage induced by HADNT with NADH and Cu(II) (Fig. 7B), suggesting that Cu(I) and H_2O_2 are essential in this reaction. DTPA also inhibited DNA damage (data not shown). Methional, a scavenger of both hydroxyl radical (·OH) and crypto-OH radicals, which mimic the free ·OH but are more discriminating in their reactions,[29,30] completely inhibited the DNA damage; additional typical ·OH scavengers (ethanol, mannitol, sodium formate, and DMSO) did not inhibit DNA damage. SOD did not demonstrate an inhibitory effect on DNA damage.

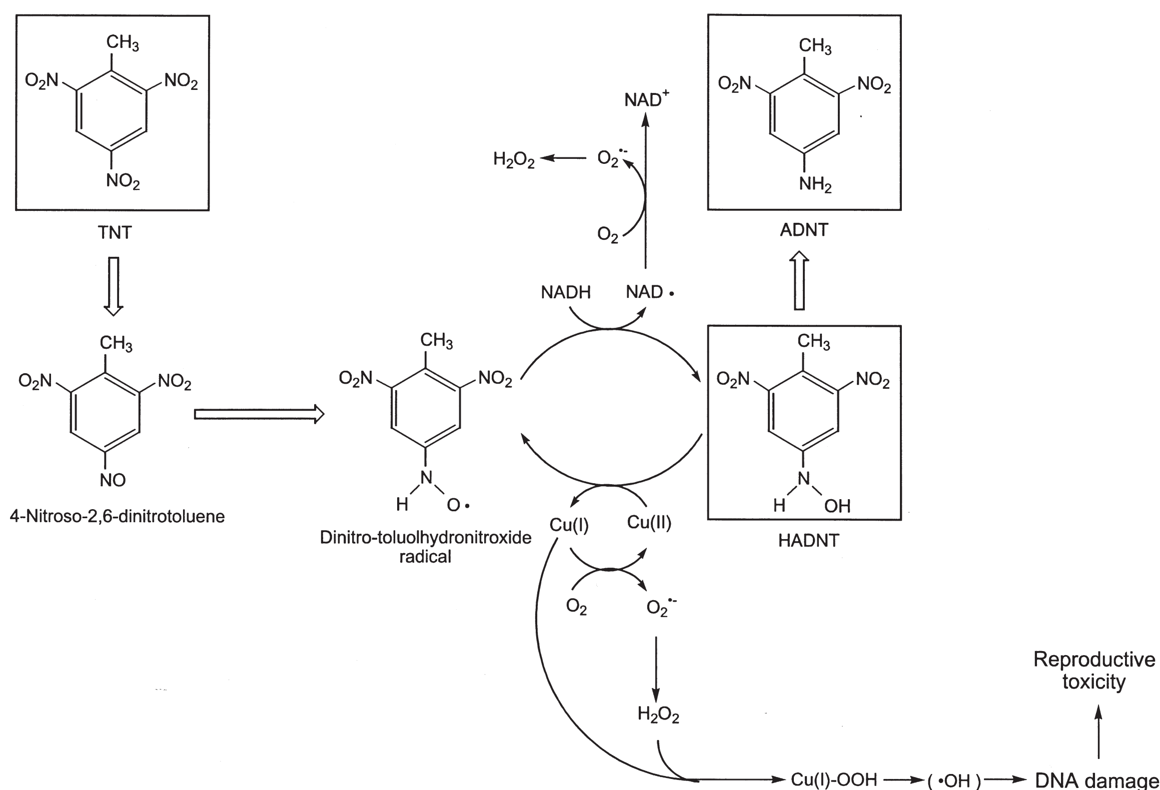

FIGURE 9 Proposed mechanism of DNA damage induced by TNT in the presence of NADH and Cu(II).

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Site Specificity of DNA Damage by HADNT

Figure 8 shows the site specificity of DNA damage induced by HADNT. HADNT caused DNA damage at consecutive guanine residues in the presence of Cu(II) and NADH. DNA cleavage was induced particularly at the 5′-GG-3′, 5′-GGG-3′ and 5′-GGGG-3′ sequences (damaged bases are underlined).

DISCUSSION

In the present study, subacute exposure of male rats to TNT resulted in a marked decrease in the weight of the testis, epididymis and seminal vesicles. On the other hand, the weight of secretory glands such as the prostate and coagulate glands was not affected. The weight changes in the reproductive organs did not recover within 2 weeks after completing the TNT-administration, while those of spleen, thymus and liver recovered. TNT induced germ cell degeneration, the disappearance of spermatozoa in seminiferous tubules, and a dramatic decrease in the sperm number in both the testis and epididymis. TNT increased the formation of 8-oxodG in sperm whereas plasma testosterone levels were not affected and no changes were observed in the size of the prostate, a testosterone-dependent organ. It is noteworthy that 8-oxodG levels in human sperm are closely associated with the impairment of sperm function and with male infertility.[31] These results suggest that TNT-induced reproductive toxicity is attributed to direct oxidative damage of spermatozoa rather than testosterone-dependent mechanisms.

To determine the mechanism of 8-oxodG formation *in vivo*, we examined DNA damage induced by TNT and its metabolic products *in vitro*. TNT is metabolized to HADNT and ADNT.[10,11] Yinon and Hwang[10] reported that ADNT is the main metabolite found in urinary samples taken from TNT workers. We revealed that HADNT, the *N*-hydroxy TNT metabolite, induced DNA damage in the presence of Cu(II) whereas TNT itself and ADNT did not. Therefore, TNT-induced reproductive toxicity may be caused by the action of the metabolite. In addition to TNT, other nitroaromatic compounds, such as dinitrotoluene and nitrobenzene, have reproductive toxic potentials.[8,32–42] Metabolic activation of these substances through nitro-group reduction[40,41] may be required for the testicular toxicity. We observed TNT accumulation in the testes and epididymides. A common mechanism appears to be involved in specific retention of nitro compounds in reproductive organs, although the mechanism has not been characterized. Interestingly, it was reported that some nitroreductase activity was detected in testicular microsomal and cytosolic fractions.[43] In this study, ADNT was detected in the testis after the final TNT injection. These results indicate that TNT-induced reproductive toxicity is attributed to oxidative DNA damage induced by its metabolite HADNT.

HADNT induced DNA damage including 8-oxodG formation in the presence of Cu(II) *in vitro*. Addition of an endogenous reductant NADH efficiently increased the DNA damage. HADNT caused site-specific DNA damage at consecutive guanine residues. We propose that HADNT undergoes Cu(II)-mediated oxidation to the dinitrotoluolhydronitroxide radical, which is then non-enzymatically reduced to HADNT by NADH (Fig. 9). Cu(I) is generated concomitantly with HADNT oxidation. Superoxide anion radical (O_2^{-}) is generated by the reaction of O_2 with Cu(I) or NAD•, and then dismutated to H_2O_2. H_2O_2 reacts with Cu(I) to form a DNA-Cu(I)-hydroperoxo complex, which may act as a crypto •OH, leading to DNA damage. This idea is supported by inhibitory effects of catalase and bathocuproine on DNA damage. Other reactive species, such as singlet oxygen ($^1O_2$) and peroxyl radicals, can cause damage to guanine but not specifically to consecutive guanines. Therefore, these species do not play an important role in TNT-induced DNA damage. It has been reported that a large part of the highest occupied molecular orbital (HOMO) is distributed on the 5′-site guanine of consecutive guanines in double-helical DNA, and thus, this guanine can be more easily oxidized than single guanine residues.[44] Similarly, metal ions bind to CG sequence more preferentially than single guanine residues.[45] Therefore, polyguanine-specific DNA damage induced by HADNT in the presence of Cu(II) may be explained by HOMO distribution. The NADH-mediated redox reaction enhances HADNT-induced DNA damage by enhancing production of reactive oxygen species. Thus, submicromolar concentrations of HADNT induced oxidative DNA damage in the presence of physiological concentrations of Cu(II) (20 μM)[46] and NADH (100 μM).[47] Therefore, it is likely that such DNA damage occurs under physiological conditions. Alternatively, it is well recognized that certain *N*-hydroxy compounds are activated by acetyltransferases and/or sulfotransferases to form DNA-bound adducts.[48,49] Therefore, the possibility that HADNT, an *N*-hydroxylated product of TNT, is metabolized to DNA-binding compounds cannot be excluded. However, in the present study, ADNT was detected at a higher level than TNT in the testis, suggesting that a substantial part of HADNT is metabolized to ADNT. Taken together, our results suggest that HADNT-induced oxidative DNA damage participates in reproductive toxicity.

Multiple nitroaromatic compounds, such as TNT, dinitrotoluene and nitrobenzene, have reproductive

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toxic potentials.[8,32–42] The present study demonstrated that a TNT metabolite, HADNT, induced Cu(II)-mediated oxidative DNA damage, whereas TNT did not. Metabolic activation via nitro-group reduction[40,41] may be prerequisite for reproductive toxicity due to these nitroaromatic compounds. Carcinogenic benzene, toluene and nitrobenzene are reproductive toxicants,[50–52] and their metabolites are also known to cause oxidative DNA damage.[42,53,54] TNT is structurally similar to the carcinogenic compounds, nitrobenzene[42] and 2,4-dinitrotoluene.[55] Epidemiological studies showed that TNT exposure was associated with liver cancer and leukemia.[56–58] These findings suggest that TNT has potential genotoxicity.

Genotoxic agents can reduce sperm counts, resulting in infertility.[59,60] Rapidly dividing germ cells, particularly spermiogenic cells, are sensitive to genotoxic insults because of their low activity of DNA repair enzymes.[8,9] In addition, the male reproductive organs are highly susceptible to oxidative damage,[31,61–63] since testes have low activities of catalase,[64,65] and spermatocytes, more differentiated germ cells, and Sertoli cells showed little or no activity of Cu,Zn-SOD.[66] In this study, we demonstrated that TNT administration decreases testicular weight and sperm number. TNT also induced an increase in 8-oxodG formation in sperm cells of the caput epididymis. This oxidative DNA damage *in vivo* may be attributed to the damage induced by a TNT metabolite as we have shown by the experiments *in vitro*. We conclude that oxidative DNA damage in the testis may play an important role in the reproductive toxicity induced by TNT and other nitroaromatic compounds.

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