Role of oxidative stress in germ cell apoptosis induced by di(2-ethylhexyl)phthalate

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Phthalate esters have been used extensively as plasticizers of synthetic polymers. Recent studies have revealed that these esters induce atrophy of the testis, although its pathogenesis remains unknown. The present study describes the possible involvement of oxidative stress in the pathogenesis of atrophy of the rat testis induced by di(2-ethylhexyl)phthalate (DEHP). Biochemical and immunohistochemical analysis revealed that oral administration of DEHP increased the generation of reactive oxygen species, with concomitant decrease in the concentration of glutathione and ascorbic acid in the testis, and selectively induced apoptosis of spermatocytes, thereby causing atrophy of this organ. Oxidative stress was selectively induced in germ cells, but not in Sertoli cells, treated with mono(2-ethylhexyl)phthalate (MEHP), a hydrolysed metabolite of DEHP. Furthermore, MEHP selectively induced the release of cytochrome *c* from mitochondria of the testis. These results indicate that oxidative stress elicited by MEHP principally injured mitochondrial function and induced the release of cytochrome *c*, thereby inducing apoptosis of spermatocytes and causing atrophy of the testis.

Key words: ascorbic acid, glutathione, phthalate esters, reactive oxygen species, testis atrophy.

INTRODUCTION

The testis is sensitive to a variety of stressors, such as hyperthermia, inflammation, radiation and exposure to agents that induce apoptosis of germ cells [1–4]. Because oxidative stress in the testis is one of the major factors that induces germ cell apoptosis, this organ has fairly high concentrations of antioxidants, such as GSH, ascorbic acid and vitamin E [5,6]. These antioxidants protect germ cells against oxidative DNA damage [7], and play important roles in spermatogenesis. In fact, deficiency of ascorbic acid and vitamin E causes the disturbance of spermatogenesis [8,9]. These facts indicate that the defence mechanism against oxidative stress plays critical roles in the maintenance of spermatogenesis and prevention of testicular atrophy. However, the mechanism by which antioxidants protect germ cells from hazardous stress, which causes atrophy of the testis, remains obscure.

Phthalate esters have been widely used in plastic devices and food packaging. These esters are found to be hazardous pollutants in biological environments [10]. In fact, a high concentration of di(2-ethylhexyl)phthalate (DEHP) has been found in the blood and tissues of patients who have received a large number of blood transfusions [11,12]. Among various phthalate esters, DEHP is one of the most widely studied toxicants in the male reproductive organs. Administration of DEHP reduces the fertility and induces testicular atrophy of laboratory animals [13–15]. When administered orally to the rat, DEHP is hydrolysed in the small intestine and other tissues by a number of esterases to produce mono(2-ethylhexyl) phthalate (MEHP) [16]. Because MEHP is the most toxic metabolite of DEHP, the monoester has been postulated to be the ultimate species responsible for the induction of atrophy of the testis [17]. Although apoptosis of spermatocytes is the underlying cause of DEHP-induced atrophy of the testis, factors that trigger the germ cell apoptosis remain unclear.

DEHP has been shown to decrease cellular levels of antioxidants [12] and increase lipid peroxidation in cultured rat hepatocytes [18]. Therefore we hypothesized that DEHP and/or MEHP might elicit oxidative stress in the testis, thereby inducing apoptosis of spermatocytes. The present study describes the changes in antioxidants and related enzymes in the testis and other tissues after administration of DEHP to the rat. The results indicate that oxidative stress elicited by MEHP derived from DEHP perturbed mitochondrial function in the testis and induced the apoptosis of spermatocytes, thereby causing atrophy of this organ.

EXPERIMENTAL

Chemicals

DEHP, GSH, 5,5′-dithiobis(2-nitrobenzoic acid), digitonin and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were purchased from Wako Chemicals (Osaka, Japan). Phthalic anhydride and 2-ethylhexanol were purchased from Aldrich (Milwaukee, WI, U.S.A.). Glutathione reductase, Cu/Zn-superoxide dismutase (SOD), catalase, ascorbic acid, sodium azide, NADH and propidium iodide were obtained from Sigma (St. Louis, MO, U.S.A.). The chemiluminescence probe L-012 {8-amino-5-chloro-7-phenylpyrido[3,4-*d*]pyridazine-1,4-(2*H*,3*H*)dione} was a gift from Takeda Chemical Industries (Osaka, Japan). All other reagents used were the highest grade commercially available.

Abbreviations used: DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DEHP, di(2-ethylhexyl)phthalate; DCF, 2′,7′-dichlorofluorescein; MEHP, mono(2-ethylhexyl)phthalate; ROS, reactive oxygen species; SOD, superoxide dismutase; TBS, Tris-buffered saline; TBST, TBS containing 0.05% Tween 20; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling.

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Synthesis of MEHP

MEHP was synthesized from phthalic anhydride and 2-ethylhexanol as described by Goggans and Copenhaver [19]. Briefly, a mixture of phthalic anhydride and 2-ethylhexanol (1:1.5, v/v) was heated for 8 h at 130 °C. After cooling, the precipitate was filtered and the filtrate was dissolved in diethyl ether. MEHP in the solution was extracted several times with an equal volume of 0.4 M potassium carbonate solution. The aqueous extract was acidified with HCl and extracted four times with diethyl ether. The diethyl ether extracts were combined, washed with water, dried with $CaCl_2$, filtered and evaporated *in vacuo*. The remaining residue was stored at −20 °C and crystallized. The crystals obtained were analysed by ^{1}H-NMR and identified as MEHP. HPLC confirmed that negligible contamination of a by-product of the reaction, phthalate, was present in the crystals.

Animals

Male Wistar rats (4–5-weeks old) were obtained from SLC (Shizuoka, Japan), fed laboratory chow and water *ad libitum*, and were used for experiments without prior fasting. Varying doses of DEHP dissolved in corn oil were administered orally using a syringe and teflon tubing. All experiments were performed according to the Guidelines for Laboratory Animal Care Regulations of Osaka City University Medical School and were approved by the Ethics Committee of the University.

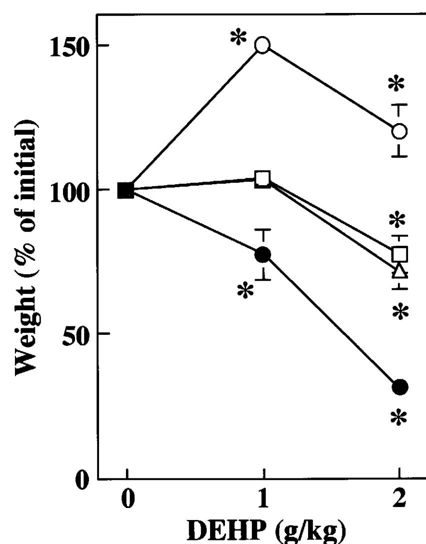

Figure 1 Effect of DEHP on tissue weight

Varying doses of DEHP dissolved in corn oil were orally administered to rats for 7 days. On day 7, the body weight (△) and testis (●), liver (○) and kidney (□) weights were determined. Corn oil (10 ml/kg) alone was administered to control animals. The whole body weight and weights of the testis, liver and kidney in control animals were 116.9 ± 2.2, 0.49 ± 0.03, 5.7 ± 0.16 and 0.56 ± 0.03 g respectively (means ± S.E.M.). Results are expressed as the percentage of initial levels and are means ± S.E.M. ($n = 8$–15). *$P < 0.05$ compared with control rats.

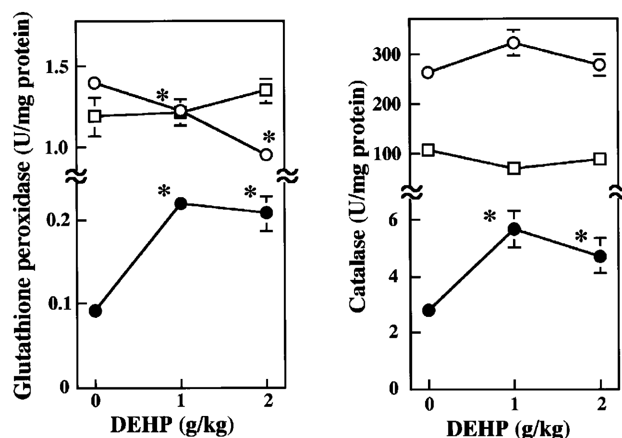

Figure 3 Effect of DEHP on glutathione peroxidase and catalase

Activities of glutathione peroxidase and catalase were determined in the testis (●), liver (○) and kidney (□) of control (rats administered with 10 ml/kg corn oil alone) and DEHP-treated rats. Other conditions were as in Figure 1. Results are expressed as means ± S.E.M. ($n = 5$). *$P < 0.05$ compared with control rats.

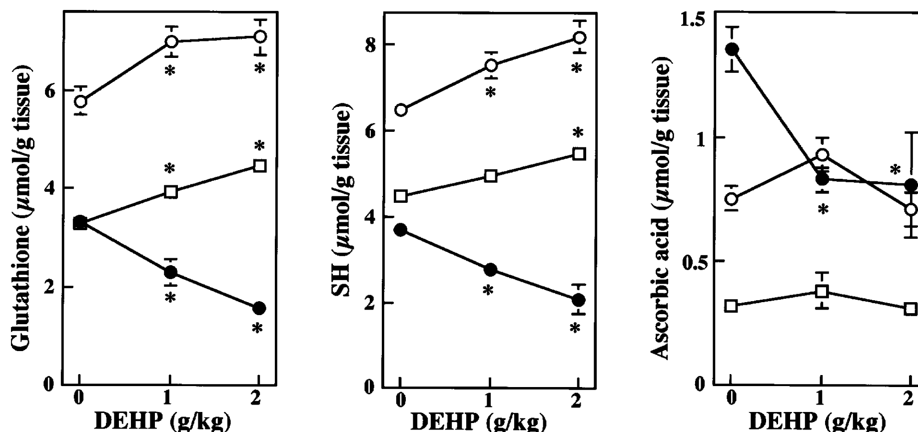

Figure 2 Effect of DEHP on tissue levels of antioxidants

Levels of total glutathione, low-molecular-mass thiols (SH) and ascorbic acid were determined in the testis (●), liver (○) and kidney (□) of control (rats administered with 10 ml/kg corn oil alone) and DEHP-treated rats. Other conditions were as in Figure 1. Results are expressed as means ± S.E.M. ($n = 8$–15). *$P < 0.05$ compared with control rats.

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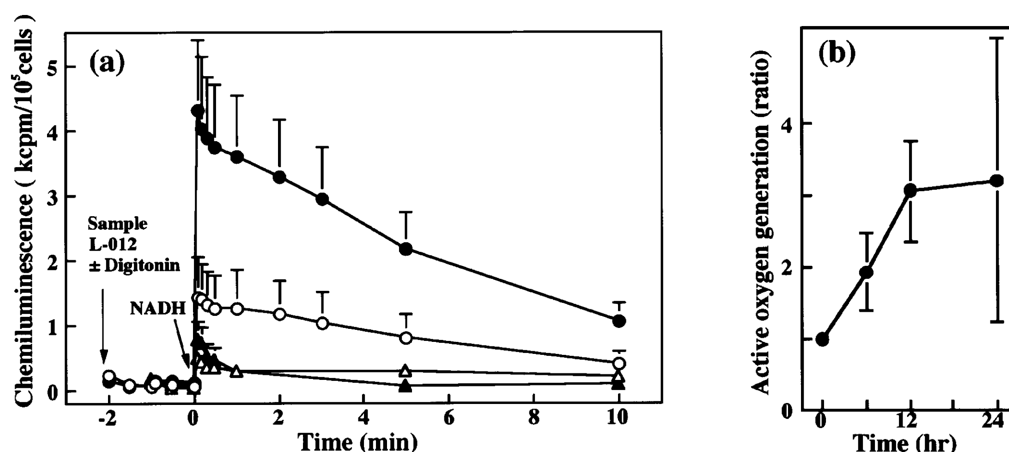

Figure 4 Effect of DEHP on the generation of ROS in the testis

(**a**) Testes were removed from rats 12 h after administration of either 2 g/kg DEHP (closed symbols) or corn oil (open symbols). Generation of ROS by testicular cells was determined in the presence (circles) or absence (triangles) of 0.005% digitonin added 2 min prior to the addition of NADH, as described in the Experimental section. Results are expressed as means ± S.E.M. ($n = 5$–9). (**b**) Rats were treated with 2 g/kg DEHP and, at the indicated times after DEHP treatment, the testes were removed. Generation of ROS by testicular cells was determined as in (**a**). Results are expressed as the relative intensity of chemiluminescence to that at time 0 and are means ± S.E.M. ($n = 5$).

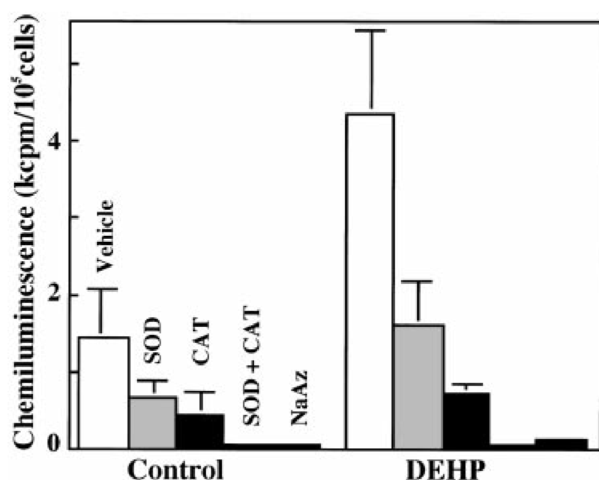

Figure 5 Effect of Cu/Zn-SOD, catalase and sodium azide on the increased generation of ROS

Testicular cells were obtained from rats 12 h after administration of either 2 g/kg DEHP or corn oil (control). Chemiluminescence intensity was determined as in Figure 4 in the absence (vehicle; open bars) or presence of Cu/Zn-SOD (SOD; 30 units/ml; hatched bars), catalase (CAT; 20 units/ml; filled bars), Cu/Zn-SOD + catalase or sodium azide (NaAz; 100 μM). The reaction was started by adding 5 mM NADH. Results are expressed as means ± S.E.M. ($n = 5$).

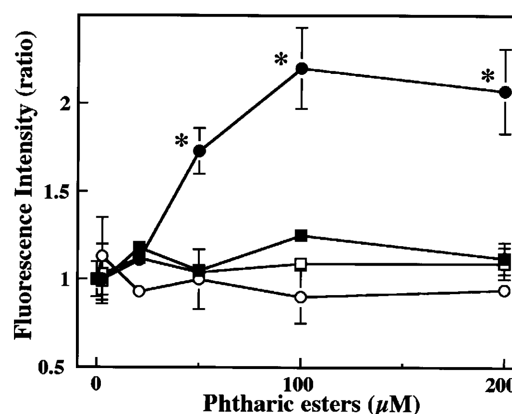

Figure 6 Effect of DEHP and MEHP on the oxidative stress in cultured testicular cells

Reactive oxygen generation by isolated germ cells (circles) and Sertoli cells (squares) was determined in the presence of either DEHP (open symbols) or MEHP (closed symbols), as described in the Experimental section. Results are means ± S.E.M. from three experiments. $^*P < 0.05$ compared with rats treated in the absence of DEHP or MEHP.

Measurement of antioxidants

Total glutathione and low-molecular-mass thiols in tissues were determined essentially as described by Tietze [20] and Ellman [21] respectively. Briefly, excised tissues were homogenized in 5 vol. of ice-cold 5% (v/v) trichloroacetic acid containing 1 mM diethylenetriaminepenta-acetic acid. After centrifugation at 10000 g for 20 min at 4 °C, low-molecular-mass thiols and total glutathione in the acid-soluble fractions were determined. Ascorbic acid in the acid-soluble fractions was determined by HPLC with electrochemical detection (Shimadzu, Kyoto, Japan), as described previously [22].

Assay for glutathione peroxidase and catalase

Fresh tissues were homogenized in 10 vol. of 10 mM potassium phosphate buffer (pH 7.4) containing 30 mM KCl and 0.1% Triton X-100. The homogenates were centrifuged at 10000 g for 20 min at 4 °C, and the supernatants were assayed for enzyme activity. Glutathione peroxidase activity was determined spectrophotometrically by monitoring the oxidation of NADPH at 340 nm [23]. Catalase activity was determined spectrophotometrically at 240 nm by the reduction of H_2O_2 as described previously [24].

Generation of reactive oxygen species (ROS) by testicular cells

Testes were removed from DEHP-treated rats under ethyl ether anaesthesia. Testicular cells were freshly obtained by mincing

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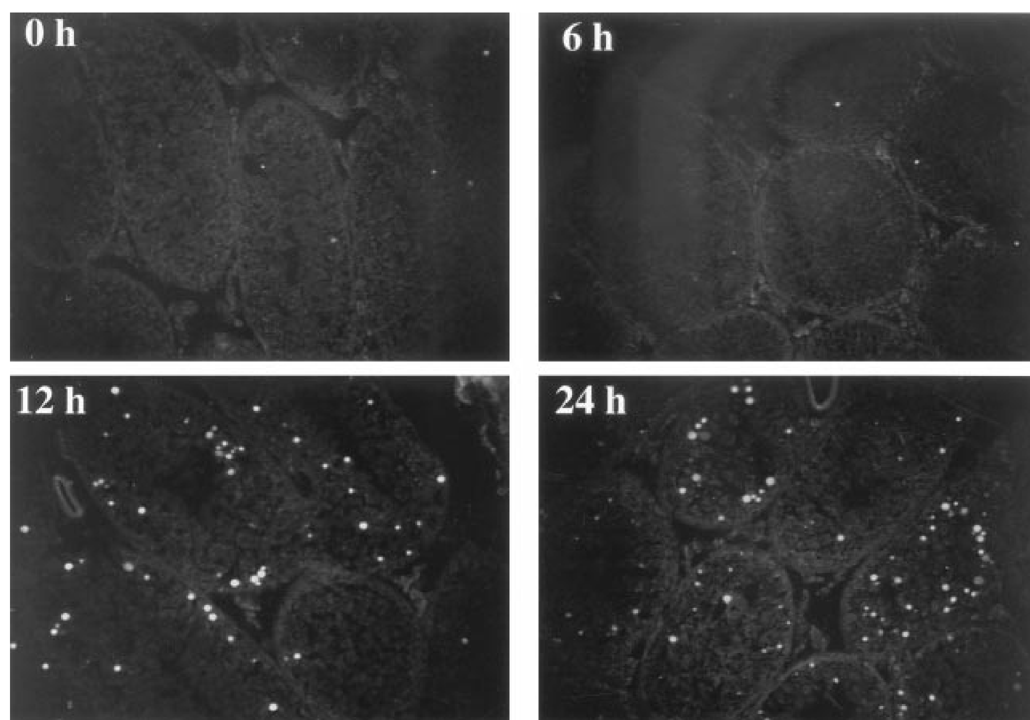

Figure 7 Effect of DEHP on the apoptosis of testicular cells

At the indicated times after oral administration of 2 g/kg DEHP, testes were removed from animals and subjected to TUNEL staining, as described in the Experimental section. Magnification, × 120.

the testis in RPMI 1640 culture medium (BioWhittaker, Walkersville, MD, U.S.A.) containing 0.1 % (w/v) type IV collagenase (Sigma), and incubating for 20 min at 32 °C essentially as described previously [25]. After differential centrifugation of the digested tissues, testicular cells were suspended in RPMI 1640 medium and filtered through a nylon mesh (20 μm in diameter) to remove cell aggregates and tissue debris. The cells obtained consisted predominantly of spermatocytes (> 90 %) as observed under a light microscope. These cells were used for biochemical analysis. Cells were incubated in 0.5 ml of PBS containing 400 μM L-012, a chemiluminescence probe [26]. After incubation with 0.005 % digitonin for 1 min at 32 °C to permeate cell membranes, the reaction was started by adding 5 mM NADH. During the incubation, chemiluminescence intensity was recorded continuously for 10 min using an Aloka Luminescence Reader BLR-201 (Tokyo, Japan).

We also analysed intracellular reactive oxygen generation by primary cultured germ cells and Sertoli cells by using the fluorescent probe DCFH-DA. Dispersed testicular cells were obtained from 14-day-old rats and plated at 10^6 cells/well in type I collagen-coated 35-mm dishes (Falcon Biocoat, Lincoln Park, NJ, U.S.A.) containing 2 ml of a 1:1 (v/v) mixture of Ham's F12 medium and Leibovitz's L15 medium (Sigma) supplemented with 1 mM L-glutamine, 10 % (v/v) foetal bovine serum, 1 g/l sodium bicarbonate, 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were cultured at 32 °C in a humidified atmosphere of 5 % CO_2 in air. After 3 days, germ cells attached to the monolayers of Sertoli cells were detached by gentle pipetting as described previously [25]. After removing the germ cells, the remaining monolayer of Sertoli cells was incubated with 2 ml of 0.25 % trypsin in PBS. The isolated germ cells and Sertoli cells were washed with PBS and incubated in PBS containing 2.5 μM DCFH-DA for 15 min. The DCFH-loaded cells (0.5×10^5 cells) were incubated with varying doses of MEHP or DEHP for 30 min. After 30 min, the fluorescence intensity of 2′,7′-dichlorofluorescein (DCF) in cells was analysed fluorometrically at 340 and 525 nm for excitation and emission respectively.

Analysis of apoptosis

Under ethyl ether anaesthesia, the testis was perfused from the abdominal aorta with 10 ml of ice-cold saline, immediately removed, embedded in Tissue-Teck OCT compound (Sakura Finetechnical, Tokyo, Japan) and stored at −80 °C. Frozen sections (5 μm thickness) were obtained using a cryostat at −20 °C, and then fixed in acetone for 15 min at 4 °C. The specimens were subjected to terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labelling (TUNEL) staining using a TaKaRa Apoptosis Detection kit (Ohtsu, Japan). Apoptosis of cells was also confirmed by staining the frozen specimens with 1 μg/ml propidium iodide for 5 min at 25 °C.

Analysis of cytochrome *c* released from mitochondria

Fresh testes were homogenized in 10 vol. of ice-cold 10 mM Tris/HCl buffer (pH 7.4) containing 0.225 M mannitol, 1 mM EDTA, 5 mg/ml BSA and 10 mM carnitine. Mitochondria were isolated from the testis as described previously [27]. Isolated mitochondria (1 mg/ml) were incubated for 5 min at 25 °C in 10 mM Tris/HCl buffer (pH 7.4) containing 0.15 M KCl and 5 mM succinate in the presence or absence of varying concentrations of either DEHP or MEHP. After incubation, each sample was centrifuged at 8000 ***g*** for 5 min at 4 °C. A portion (50 μl) of the supernatants was dissolved in 0.1 ml

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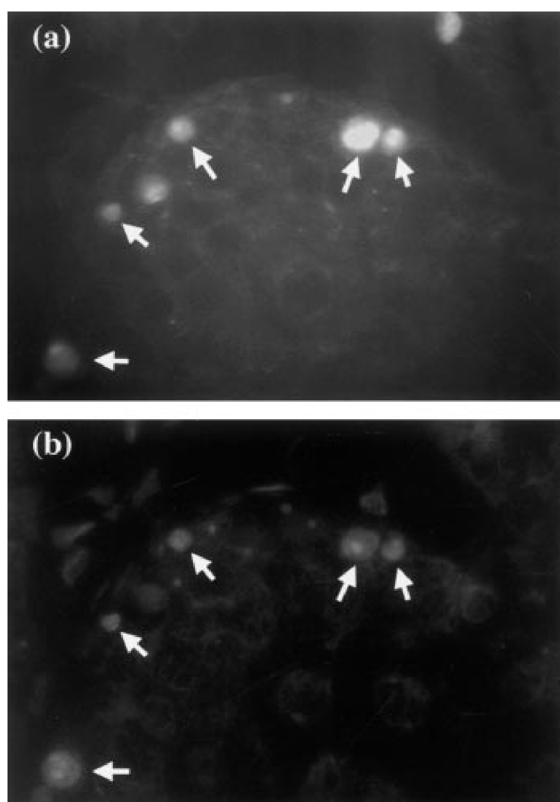

Figure 8 TUNEL and propidium iodide staining in DEHP-treated rat testis

After 12 h of DEHP administration (2 g/kg), testes were excised and subjected to TUNEL (**a**) and propidium iodide (**b**) staining. TUNEL-positive spermatocytes were also stained by propidium iodide (arrows). Magnification, $\times 650$.

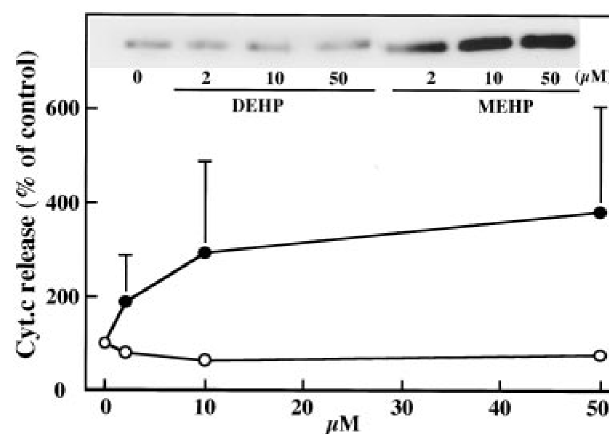

Figure 9 Effect of DEHP and MEHP on cytochrome *c* release from mitochondria

Mitochondria (1 mg/ml) isolated from the testis were incubated in 10 mM Tris/HCl buffer (pH 7.4) containing 0.15 M KCl, 5 mM succinate and varying concentrations of either DEHP (○) or MEHP (●) for 5 min at 25 °C. After incubation, the mixtures were centrifuged at 8000 ***g*** for 5 min at 4 °C. Portions of the supernatants were subjected to SDS/PAGE followed by Western-blot analysis. The percentage of cytochrome *c* released following treatment (means $\pm$ S.E.M., $n = 3$), expressed as a percentage of control levels, is shown along with a representative Western blot of cytochrome *c* levels.

of 125 mM Tris/HCl buffer (pH 7.4) containing 4 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol, 20 % (v/v) glycerol and 0.002 % Bromophenol Blue. After boiling at 100 °C for 5 min, the samples were subjected to SDS/PAGE followed by Western-blot analysis.

Western blotting

Electrophoresed proteins were transferred from the gel on to an Immobilon membrane (Millipore, Waltham, MA, U.S.A.). The membrane was blocked in Tris-buffered saline [TBS; 10 mM Tris/HCl buffer (pH 7.4) and 0.15 M NaCl] containing 5 % (v/v) skimmed milk, and then incubated with primary antibody diluted with TBS containing 0.05 % Tween 20 (TBST). The membrane was then incubated for 1 h at 25 °C with peroxidase-conjugated secondary antibody (DAKO, Glostrup, Denmark) diluted with TBST containing 5 % (v/v) skimmed milk. Immunoreactive bands were visualized using ECL® (Amersham Biosciences, Uppsala, Sweden).

Statistics

Data are expressed as the mean $\pm$ S.E.M. derived from 5–15 animals. Statistical analysis was performed using ANOVA followed by Student's *t* test, and the level of significance was set at $P < 0.05$.

RESULTS

Effect of DEHP on tissue and body weights

Administration of DEHP for 7 days induced a dose-dependent decrease in testis weight (Figure 1). At a dose of 1 g/kg, DEHP increased the weight of liver without affecting the body weight of the rat. However, DEHP started to decrease the weight of kidneys and whole body weight at doses higher than 1 g/kg.

Effect of DEHP on the antioxidant status in various tissues

To test the possible involvement of oxidative stress in the pathogenesis of testis atrophy, we determined the concentrations of total free thiol, GSH and ascorbic acid in the testis, liver and kidney of DEHP-administered rats. DEHP decreased testis levels of free thiol, GSH and ascorbic acid in a dose-dependent manner. In contrast, DEHP increased the levels of free thiols and GSH in both liver and kidney (Figure 2). Under identical conditions, plasma and brain levels of these antioxidants remained unchanged during the experiments (results not shown). We also measured changes in the activity of glutathione peroxidase and catalase in tissues after treating animals with DEHP. The activities of both enzymes increased in the testis, but not in liver and kidney of DEHP-treated animals (Figure 3). Hepatic activity of glutathione peroxidase decreased slightly.

Effect of DEHP on the generation of ROS in the testis

The decrease in antioxidant levels and increase in antioxidant enzymes in the testis of DEHP-treated rats appeared to reflect the increase in oxidative stress. Thus we analysed the generation of ROS in testicular cells after administration of DEHP. In the presence of digitonin, the intensity of L-012-dependent chemiluminescence increased after adding 5 mM NADH (Figure 4). The chemiluminescence intensity was stronger with testicular cells obtained from DEHP-treated animals than from the control group. Furthermore, the chemiluminescence increased

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in a time-dependent manner after administration of DEHP. To elucidate the chemical species responsible for the increased chemiluminescence, Cu/Zn-SOD and/or catalase were added to the reaction mixture to scavenge superoxide and H_2O_2 respectively (Figure 5). The chemiluminescence intensity decreased significantly in the presence of either Cu/Zn-SOD or catalase. In the presence of both Cu/Zn-SOD and catalase, the L-012-dependent chemiluminescence disappeared completely. Furthermore, the presence of sodium azide also inhibited the L-012-dependent chemiluminescence in a manner similar to Cu/Zn-SOD plus catalase.

Effect of DEHP and MEHP on oxidative stress in isolated testicular cells

Effects of MEHP and DEHP on oxidative stress in isolated germ cells and Sertoli cells were determined using DCFH-DA (Figure 6). MEHP, but not DEHP, increased the fluorescence intensity of DCF derived from germ cells in a dose-dependent manner. In contrast, neither DEHP nor MEHP affected the fluorescence intensity of Sertoli cells.

Effect of DEHP on the fate of testicular cells

To test the possible involvement of apoptosis in the mechanism of DEHP-induced atrophy of the testis, we examined the occurrence of DNA fragmentation in the testis using TUNEL staining. The number of TUNEL-positive cells in the testis markedly increased 12 h after the administration of DEHP (Figure 7). Most of the TUNEL-positive cells in the seminiferous tubules were identified primarily as spermatocytes. Because the specificity of TUNEL staining as a marker of apoptosis is low, we also studied the possible occurrence of apoptosis using propidium iodide (Figure 8). The TUNEL-positive germ cells also showed chromatin condensation (arrows in Figure 8), suggesting that TUNEL-positive cells are apoptotic cells.

Effect of DEHP and MEHP on cytochrome *c* release from mitochondria

The release of cytochrome *c* from mitochondria into the cytosol is one of the key factors responsible for inducing apoptosis in various cell types. Therefore we examined the effect of DEHP and MEHP on cytochrome *c* in mitochondria. Figure 9 shows that fairly low concentrations of MEHP, but not DEHP, induced the release of cytochrome *c* from mitochondria.

DISCUSSION

The present study indicates that administration of DEHP increased the generation of ROS and selectively decreased GSH and ascorbic acid in the testis, thereby inducing apoptosis of spermatocytes to cause atrophy of this organ.

Administration of DEHP induced hypertrophy of the liver. Because intestinal esterases rapidly hydrolyse DEHP to MEHP [16], this metabolite might accumulate in liver via the organic anion transport system [28]. MEHP has a potent activity to stimulate the proliferation of peroxisomes [29,30], whereas administration of DEHP stimulates Kupffer cells to synthesize and secrete mitogenic cytokines [31]. Presumably, due to such properties of the phthalates, they would have induced the proliferation of hepatocytes to cause hypertrophy of the liver.

Because mild oxidative stress increases hepato-renal GSH levels [32] and MEHP enhances the generation of ROS in mitochondria, this metabolite might be responsible for the observed increase of GSH in the liver and kidney. At doses of DEHP higher than 1 g/kg, not only the weight of the testis, but also that of the kidney and whole body decreased, suggesting the toxic effect of a high dose of the phthalate. DEHP increased the activity of glutathione peroxidase and catalase in the testis without affecting catalase activity in liver and kidney. Thus the sensitivity of the testis, liver and kidney to DEHP and/or MEHP seems to differ from one organ to another. Because germ cells showed high sensitivity to MEHP and selectively underwent apoptosis, the increased activity of glutathione peroxidase and catalase in the testis may reflect their levels predominantly in the remaining cells, such as Sertoli cells.

The present study also demonstrates that DEHP enhanced the generation of ROS by testicular cells. Kinetic analysis using Cu/Zn-SOD and catalase revealed that the superoxide radical and H_2O_2 were responsible for the increased generation of reactive species. Low concentrations of MEHP, but not DEHP, induced the release of cytochrome *c* from testis mitochondria, a key step in the induction of apoptosis [33]. MEHP is the major metabolite found in the testis of DEHP-administered rats [17,34]. After administration of 2 g/kg DEHP into the rat, approx. 100 μmol/kg of MEHP was found to accumulate in the testis [35]. Because this concentration of MEHP is sufficient to inhibit state III respiration and thus increase the generation of the superoxide radical [35,36], oxidative stress elicited in and around mitochondria is proposed to have induced the release of cytochrome *c* from the mitochondria to cytosol in the testis of DEHP-treated animals. The previous observation [37] that the DEHP-induced atrophy of the testis was inhibited successfully by administration of ascorbic acid and vitamin E is consistent with our hypothesis described above.

Administration of DEHP induces vacuolation in Sertoli cells [34]. Under the present experimental conditions, vacuolation in Sertoli cells of the DEHP-treated rat testis was also observed by electron microscopy (results not shown). Thus DEHP and/or MEHP might also affect the functions of Sertoli cells. In this context, MEHP has been shown to up-regulate the expression of Fas ligand in Sertoli cells [38,39]. Furthermore, administration of MEHP to *gld* mice, which express a non-functional Fas ligand, failed to induce germ cell apoptosis [40]. These results suggest that the expression of Fas ligand in Sertoli cells might also play a role in the pathogenesis of MEHP-induced apoptosis of spermatocytes. The promoter region of the gene encoding Fas ligand involves the binding site for nuclear factor-κB ('NF-κB'), the function of which is redox regulated [41–43]. Because administration of DEHP activates nuclear factor-κB in Kupffer cells, a similar activation is likely to have occurred in Sertoli cells to up-regulate their Fas ligand, thereby triggering the reaction leading to germ cell apoptosis.

DEHP-induced apoptosis in the testis was observed primarily with pachytene spermatocytes, but not with Sertoli cells. Because Sertoli cells contain higher concentrations of GSH, vitamin E and GSH-related enzymes than pachytene spermatocytes and round spermatids [44,45], differences in their susceptibility to phthalate toxicity may be explained by the difference in their activities of antioxidants and related enzymes. Thus the enhanced release of cytochrome *c* in spermatocytes and activation of the Fas/Fas ligand system in Sertoli cells may co-operate in inducing apoptosis of germ cells in the testis.

Testosterone is the critical hormone that maintains spermatogenesis in the testis. Although administration of DEHP decreased the testosterone levels in testes and plasma, loading doses of testosterone failed to inhibit the DEHP-induced

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testicular atrophy in the rat [46]. Thus testosterone may not be involved in the pathogenesis of DEHP-induced atrophy in the testis.

Apart from the mechanism of spermatocyte apoptosis, selective killing of injured germ cells might play an important role in eliminating defective spermatocytes and increasing the efficacy of fertilization by intact cells. The molecular mechanism for the selective induction of apoptosis in testicular germ cells by DEHP and related compounds requires further elucidation.

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