Possible Origin of Rat Testicular Atrophy Induced by Di-*n*-Butyl Phthalate: Changes in the Activities of Some Enzymes during Rat Testis Perfusion under a Hypoxic Condition and with Mono-*n*-Butyl Phthalate

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To examine whether testicular toxicity in rats is caused by a direct effect of mono-butyl phthalate (MBP), a metabolite of di-butyl phthalate, or by a secondary effect attributed to a hypoxic condition due to the MBP-induced hemoglobin deprivation, the testes were perfused with a solution of MBP in Eagle's MEM or the MEM with/without oxygen, and the activities of testicular enzymes were measured. A decrease in the succinate dehydrogenase (SUDH) activity was observed by the hypoxic perfusate [20–30% dissolved oxygen (DO)], and an induction of apoptosis was observed by the 7% DO perfusate. However, the 100 mM MBP perfusate decreased the activity of SUDH per testis weight, but not per protein level. Therefore, this study proposes that the toxicity might be caused by hypoxia and a coincident depletion of SUDH activity, followed by an apoptotic testicular cell death.

Key words —— di-*n*-butyl phthalate, mono-*n*-butyl phthalate, succinate dehydrogenase, testicular damage, testicular perfusion, hypoxia

INTRODUCTION

Phthalates are used in the manufacture of flexible plastics. Recent interest in phthalates has focused on influence for male gonads. Over the past several years, a number of reviews concerning the reproductive toxicity of phthalates have been reported. In them it is concluded that no direct evidence links human exposure to phthalates, based mainly on lack of information.[1–4] Foster *et al*.[5] have reported that human exposure data would indicate worst-case scenarios to infants in the dose range of 66 μg/kg/day, and that a default risk assessment appeared to be inappropriate, since rodents, unlike primates, metabolize phthalate diesters (di-butyl- and di-2-ethylhexyl-phthalate) to monoesters extensively in the gut following oral administration. Toxic effects of phthalates in the animal model have been characterized by changes in blood cell and sperm counts and in testicular enzyme activities, as well as by anti-androgenic effects and testicular atrophy in many reviews. Foster *et al*.,[5] Fukuoka *et al*.,[6–9] and Oishi and Hiraga[10] have addressed the strong evidences that the monoester is the active principle in inducing reproductive toxicity. We know relatively little about the toxic mechanism involved in time points by di-butyl phthalate (DBP) and/or its metabolite, mono-butyl phthalate (MBP), through which testicular damage is characterized by the sloughing of germ cells in histological findings, following decreases in the testicular levels of enzymes [including succinate dehydrogenase (SUDH)] at toxic doses at which the damage was monitored by testicular atrophy observed within one week after oral DBP-treatment.[5–7,11,12] A decrease in iron levels of both the blood and red blood cells (RBC) was observed to coincide with or prior to the decrease in SUDH activity of the Sertoli cells, accompanied by a decrease in the iron level of interstitial cells and an increase in the activity of lactate dehydrogenase (LDH) in the germ cells.[8,9,12]

In the primary culture,[13,14] germ cell detachment from the Sertoli cells was demonstrated at 10^{-7} or

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10^{-6} M of mono-2-ethylhexyl phthalate (MEHP), a metabolite of di-2-ethylhexyl-phthalate. The dose corresponded to femto (f) mol per the Sertoli cell, indicating a 10^2 or 10^3-fold concentration [atto (a) mol/cell] in the Sertoli cell *in vivo* study,[12] because the total numbers[15] of the germ and Sertoli cells were about 92×10^6 and 16×10^6 per rat testis, respectively. Furthermore, Richburg and Boekelheide[16] have reported that MEHP exhibits apoptosis on the germ cells. Accordingly, the toxic mechanism should be investigated using *in vivo* dose levels[6–9,12] in the animal model in order to investigate further the health hazard potential in humans.

However, questions remain about the toxic mechanism: whether these phenomena are caused directly by the monoesters including MBP and MEHP, or secondarily by the hypoxia due to hemoglobin deprivation induced by the metabolite. Preliminary to solving it, the testes were perfused with a solution of MBP in Eagle's MEM or MEM with/without oxygen. The toxic mechanisms were determined by changes in the activities of testicular enzymes and by apoptosis of the testicular cells. Moreover, it was determined whether an oral dose of DBP induced the apoptosis in rat testes.

MATERIALS AND METHODS

Chemicals —— Mono-*n*-butyl phthalate (MBP; purity 99.9%) was synthesized by heating an equivalent mixture of phthalic anhydride and *n*-butyl alcohol at 120ºC for 40 min, according to the known method.[14] Eagle's MEM and L-glutamine were purchased from Nissui Pharmaceuticals and GIBCO BRL (MD, U.S.A.), respectively. A Bio-Rad Protein Assay kit and pentobarbital were from Bio-Rad and Dainippon Pharmaceuticals, respectively. A sorbitol dehydrogenase assay kit was from Sigma-Aldrich Fine Chemicals (U.S.A.). A DNA standard molecular markers set (1 kb Molecular Ruler) was from Amersham-Pharmacia Biotech (Japan). Fe-Kainos and Fe-Mono Kainos for iron assay kits were from Kainos (Tokyo, Japan). A testosterone EIA kit was from Cayman Chemical Company (U.S.A.). Di-*n*-butyl phthalate (DBP; purity 99.9%), other reagents and other assay kits were from Wako Pure Chemicals (Tokyo, Japan).

Partition Coefficient of MBP —— The coefficient was obtained from a partition of MBP at a ratio of *n*-octanol to water: a solution (5 μl) of MBP (10 mg) in dimethyl sulfoxide (1 ml) was added into the mixture-solution of *n*-octanol saturated with water (1 ml) and each (1 ml) of 0.1 M acetic acid (pH 3.0), 0.1 M acetate buffer (pH 4.5), and 0.1 M phosphate buffer (pH 5.6, 6.5 and 7.4), respectively. The mixture was mixed vigorously for 3 min and was allowed to stand for 3 hr. MBP concentration in the aqueous layer was analyzed by a Shimazu UV spectrophotometer (Model UV 2200) at 280 nm.

Concentration of MBP in the Testis Perfusion —— It was necessary to provide an MBP concentration in the perfusate of at least more than 10 times the MBP concentration in the testis. Partition coefficients of MBP were 34.6, 4.61, 0.95 and 0.12 at pH 3.0, 4.5, 6.5 and 7.4, respectively, and the coefficient of 0.12 at pH 7.4 represented 12% permeability from the blood to tissues and/or organs. MBP concentration in the testis was observed at 114.4 ± 5.4 nmol/g testis at 3 hr after an orally toxic dose of 8.6 mmol DBP/kg,[12] and the total volume of blood in the rat testis was about 10 μl/g.[18] Therefore, MBP was provided at more than 10 mM in the testis and 100 mM in the perfusate.

Perfusion Method of the Testis —— MBP was dissolved in a solution of Eagle's MEM (pH 7.4, including 2 mM glutamine) at various concentrations: 1, 10 and 100 mM. The perfusate was bubbled with $95\%O_2$–$5\%CO_2$ completely. A hypoxic perfusate was bubbled with N_2 gas, and the O_2 content in the perfusate was monitored by dissolved oxygen (DO) using DO-21P (Toa Electronics, Japan).

Adult male Wistar rats (Charles River, Yokohama, 5 weeks old, weight range, 185–230 g) were treated after acclimatization for one week in this study. Rats were anesthetized with pentobarbital, then a perfusion-testis operation was performed: the abdominal aorta was ligated at the renal branches, a silicon tube was inserted and ligated in the junction separating the right and left common iliac arteries, and the lumbar, mesenteric and renal arteries were ligated. The perfusate was pumped into the tube at the rate of 5 ml/min at 25ºC using a peristaltic pump (Iwaki PST-200). Blood and the perfusate were excreted from the common iliac vein, respectively. After 4 hr-perfusion, the testis was removed from the body, weighed immediately, minced in cold phosphate buffer [10 mM, pH 7.0, 2 vol (g/ml)], homogenized at 250–260 rpm for 3 min on the ice bath, and centrifuged at $960 \times g$ at 4ºC for 15 min to give a supernatant. The supernatant was used to measure activities of the enzymes, as well as adenosine triphosphate (ATP), iron, protein and testosterone levels.

Table 1. Rat Body and the Perfused Right Testis Weights

perfusion	BW (g)	testis weight (g)	testis/BW $\times 10^{-3}$
0 hr perfusion	190.0± 8.7	1.03±0.03	5.42±0.2
4 hr perfusion			
control	194.0±15.6	1.18±0.04	6.08±0.5
10 mM MBP	211.7±16.9	1.21±0.19	5.72±0.7
100 mM MBP	190.0±25.0	1.20±0.18	6.31±0.9
20–30% DO	210.0±18.0	1.14±0.21	5.43±0.3
< 7% DO	195.0±14.1	1.14±0.17	5.83±0.3

The results are means ± S.D. for 5 rats. BW means body weight.

Measurement of ATP, Enzyme Activity, Iron, Protein and Testosterone ——

ATP Assay: A homogenate of the perfused testis was centrifuged at 960 × *g* for 15 min followed by recentrifugation at 8000 × *g* for 10 min at 4ºC to give a supernatant. The ATP level in the supernatant was assayed using a TOA ATP Analyzer AF 100 (TOA Electronics, Ltd., Japan) equipped with a TOA Auto Dispenser DF 10.

Enzyme Activity Assay: The targeted enzymes were those that showed changes in their activities before the dissociation of germ cells.[6] The enzyme activities of SUDH and aldose reductase (ADR) were assayed by the methods of Pennington[19] and Tanimoto *et al*.,[20] respectively. The activities of lactate dehydrogenase (LDH), γ-glutamyl transpeptidase (γ-GTP) and alkaline phosphatase (ALP) were determined with commercially available assay kits. The enzyme activity in each sample was assayed in duplicate to obtain the mean value.

Iron Assay: The total iron in the perfused testis homogenates was determined by an assay kit, Fe-Mono Kainos (Kainos).

Protein and Testosterone Assays: The assays were carried out using two assay kits, Bio-Rad Protein Assay Kit and Testosterone EIA Kit, respectively.

Apoptosis of Testicular Cells and Changes in Testicular Levels of SUDH after Dosing with 3-Nitropropionic Acid (3-NPA) or DBP —— Gould *et al*.[21] and Wang *et al*.[22] reported that 3-nitropropionic acid (3-NPA) inhibited SUDH (induced ATP depletion). To examine whether 3-NPA induced apoptosis and the depletion of SUDH activity (ATP depletion) in the testis, 3-NPA was injected to 5-week-old rats (body weight 145–155 g) at 15 mg/kg subcutaneously, and DNA fragmentation and SUDH (ATP) depletion were examined at 12 hr after the dosing.

A single oral dose of DBP (8.6 mmol/kg) was administered to rats, and apoptosis and depletion of SUDH activity were examined at 12 hr after the dosing, according to the method described by Fukuoka *et al*.[6] and Zhou *et al*.[12]

DNA Gel Electrophoresis —— The detection of low molecular weight DNA ladders was performed using an Apoptosis Ladder Detection Kit Wako (Wako Pure Chemicals, Tokyo, Japan): The extracted DNA was dissolved in TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0) (50–100 μl). Aliquots of DNA (3.0 to 3.8 μg DNA/well) were loaded onto 2% agarose gels and separated by electrophoresis (50 V, 1.5 hr). DNA was stained with SYBR Green and visualized with an ultraviolet transilluminator (Model NTM-10; Funakoshi, Japan) at 302 nm. The sizes of resulting DNA bands were estimated by comparison with standard molecular markers.

A positive control testicular DNA laddering pattern was prepared according to the method by Lee *et al*.[23]

Statistical Analyses —— Data are expressed as the mean ± S.D. (n = 5). Data for control and treatment groups were compared using the unpaired Student's t-test (two-tailed) or the Dunnett-type mean rank test.[24]

RESULTS

Levels of Proteins, Enzyme Activities, ATP, Testosterone and Iron in the Perfused Testis

Table 1 shows the body weight of rats used and testis weight after perfusion in the study. There was no significant difference in the ratios of testis weights to body weights among the perfusion groups: 0 hr perfusion, 4 hr perfusion with control, 10 mm MBP, and 100 mM MBP, or under hypoxic conditions (20–30% DO and < 7% DO) .

When a control perfusate was circulated in the testis, the activities of testicular enzymes were not

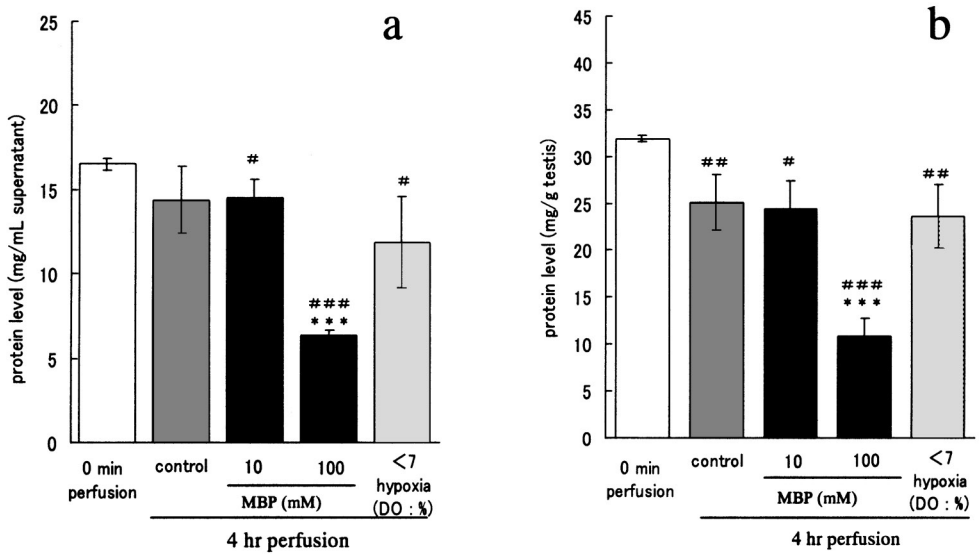

Fig. 1. Protein Level in the Perfused Testis

a: levels per supernatant of perfused testicular homogenate, b: level per g perfused testis. Values are mean ± S.D. for 3 or 5 rats. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.005$ compared with the 0 hr perfused control. ***$p < 0.005$ compared with the 4 hr perfused control. DO is % of air (about 8.3 mg/l, 37ºC. DO < 7% is corresponding to < 0.58 mg/l.

constant during the 30 min perfusion because of incomplete removal of constituents from the testicular blood vessels, but they were then constant from 1 to 4 hr. The 0 time means the time at which blood was exchanged with the perfusate in the testis.

Protein levels were measured using homogenates of the perfused testes (Fig. 1). Protein levels in the control perfused testes decreased when compared with the protein levels of the 0 time perfused testis. When protein levels were compared between the control perfused testis and the treated MBP or hypoxic testis, a decrease was observed at 100 mM MBP but not under a hypoxic condition. The decrease in its ratio to testis weight was conceivable to be related to the removal of constituents in the blood vessels during 4 hr of perfusion.

Changes in the activities of enzymes were observed at 4 hr perfusion (Figs. 2–6). When enzyme activities in the control perfused testes were compared with those in the 0 time testis, the activities of SUDH, γ-GTP and ADR did not change, but ALP increased per protein level and testis weight, and LDH decreased per testis weight.

When MBP perfused or hypoxic testis were compared with the 4 hr perfused controls, the activities per mg protein of all the target enzymes increased at 100 mM MBP (Figs. 2–6), but only the SUDH activity per protein decreased in hypoxia (Fig. 2a).

The activity per testis of SUDH decreased at both 100 mM MBP and hypoxia (DO: 20–30%) (Fig. 2), and the activities of LDH and ADR decreased at 100 mM MBP (Figs. 4 and 6). The activities of ALP and λ-GTP increased or did not change at 100 mM MBP, respectively (Figs. 3 and 5).

As for the levels of ATP, iron and testosterone, ATP levels per protein and testis increased at 100 mM MBP and at hypoxia (DO: 7%)(Fig. 7), respectively, when compared with the 4 hr perfused controls. Iron levels did not change. The testosterone level per testis showed an ascending tendency at hypoxia (DO: 7%), but not at 100 mM MBP (Fig. 8).

Changes in Testicular Levels of SUDH after Dosing of 3-NPA or DBP

Since it was reported that 3-NPA was an inhibitor of SUDH,[21,22] 3-NPA was used to determine whether SUDH depletion induced apoptosis. Testis weights of rats treated with 3-NPA or DBP were not different from those of the controls (579 ± 20 mg), whereas SUDH activities showed a significant depletion 2.04 ± 0.2 mIU/mg protein ($p < 0.05$) and 87.8 ± 13.9 mIU/g testis in the rats treated with 3-NPA, and there were significant decreases ($p < 0.05$), 503 ± 0.1 mg and 2.33 ± 0.2 mIU/mg protein, respectively, by comparison with those of the controls (2.90 ± 0.3 and 112.8 ± 9.0) (Table 2).

DNA Gel Electrophoresis Analysis

Apoptosis is also characterized by low molecu-

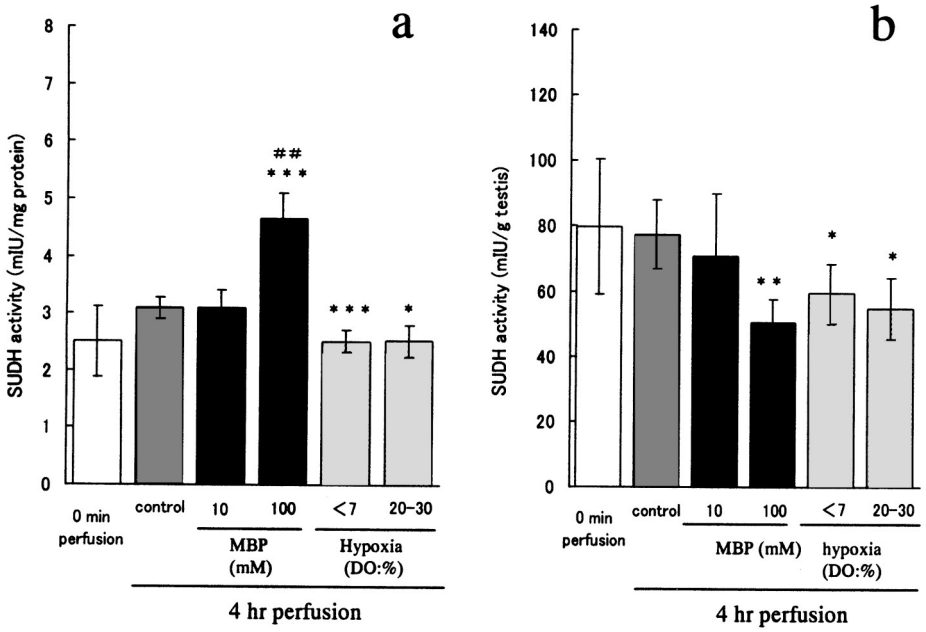

Fig. 2. SUDH Activity in the Perfused Testis

a: activity per total protein, b: activity per g perfused testis. Values are mean ± S.D. for 5 rats. ##$p < 0.01$ compared with the 0 hr perfused control. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ compared with the 4 hr perfused control. DO is % of air (about 8.3 mg/l, 37ºC). DO < 7% is corresponding to < 0.58 mg/l and DO 20–30% is 1.66–2.49 mg/l.

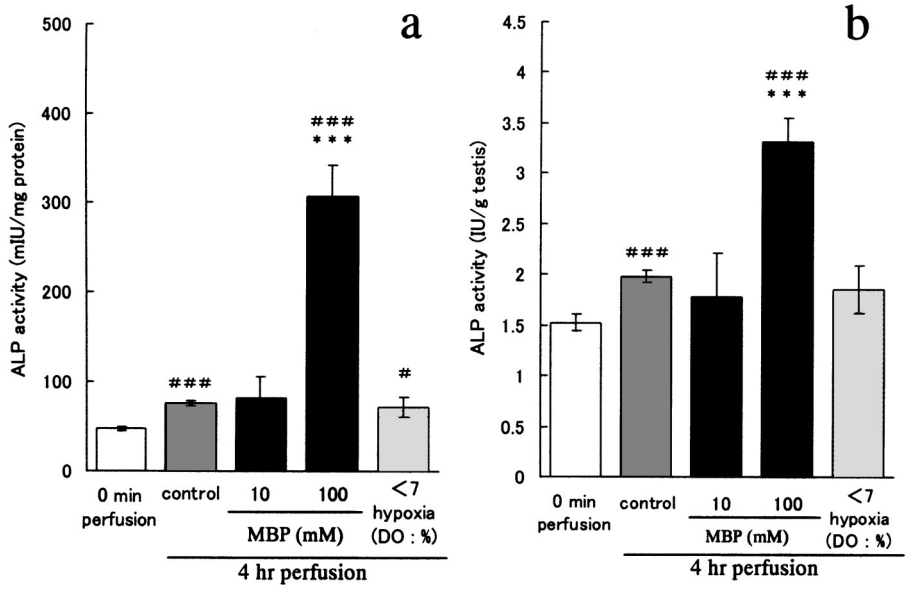

Fig. 3. ALP Activity in the Perfused Testis

a: levels per total protein, b: level per g perfused testis. Values are mean ± S.D. for 5 rats. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.005$ compared with the 0 hr perfused control. **$p < 0.05$, ***$p < 0.005$ compared with the 4 hr perfused control. DO is % of air (about 8.3 mg/l, 37ºC). DO < 7% is corresponding to < 0.58 mg/l.

lar weight DNA fragments that give a laddering pattern after gel electrophoresis. The DNA fragmentation pattern was examined both in the testes of the control and of the 4 hr-perfusion with 100 mM MBP and with hypoxia at lower than 7% and at 20–30% DO, as well as in the testes of the control, 3-NPA and DBP treated rats. DNA ladders were observed for the testes from a hypoxic (lower 7% DO) perfusate and from the DBP treated rats, but not for the other testes.

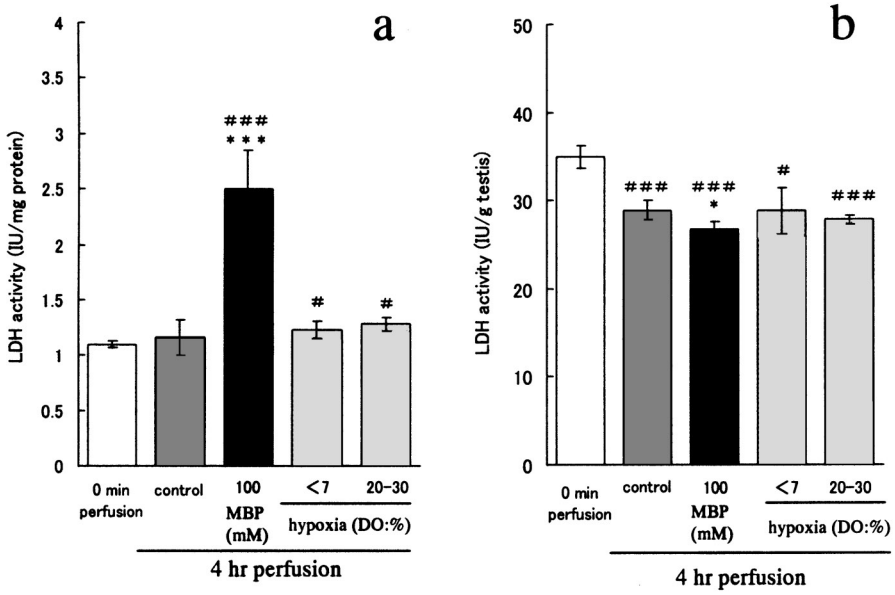

Fig. 4. LDH Activity in the Perfused Testis

a: activity per total protein, b: activity per g perfused testis. Values are mean ± S.D. for 5 rats. #$p < 0.05$, ###$p < 0.005$ compared with the 0 hr perfused control. *$p < 0.05$, ***$p < 0.005$ compared with the 4 hr perfused control. DO is % of air (about 8.3 mg/l, 37°C). DO < 7% is corresponding to < 0.58 mg/l and DO 20–30% is 1.66–2.49 mg/l.

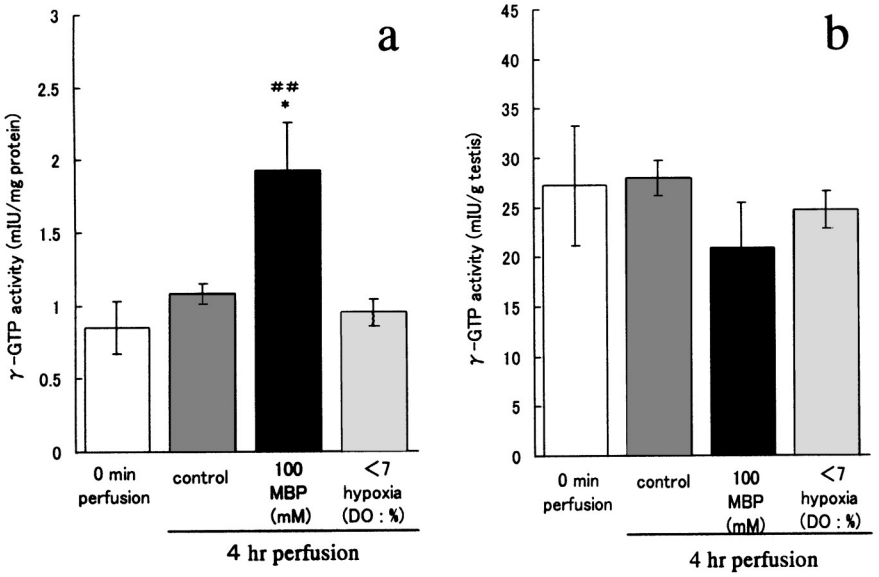

Fig. 5. γ-GTP Activity in the Perfused Testis

a: activity per total protein, b: activity per g perfused testis. Values are mean ± S.D. for 5 rats. ##$p < 0.01$ compared with the 0 hr perfused control. *$p < 0.05$ compared with the 4 hr perfused control. DO is % of air (about 8.3 mg/l, 37°C). DO < 7% is corresponding to < 0.58 mg/l.

DISCUSSION

Relative to the reproductive toxicity by DBP, Fukuoka *et al.*[6–9] proposed a possible mechanism in time sequences of toxic events by which DBP was metabolized to MBP, and MBP might then pass through the erythrocyte membrane to accelerate auto-oxidation of hemoglobin (Hb), accompanied by the depletion of glutathione (GSH) and the release of iron, making the erythrocytes transporters of low oxygen. Consequently, MBP caused a sloughing of mature germ cells in the early stages of damage, followed by atrophy with the dissociation of germ cells from the Sertoli cells. Before the sloughing, MBP

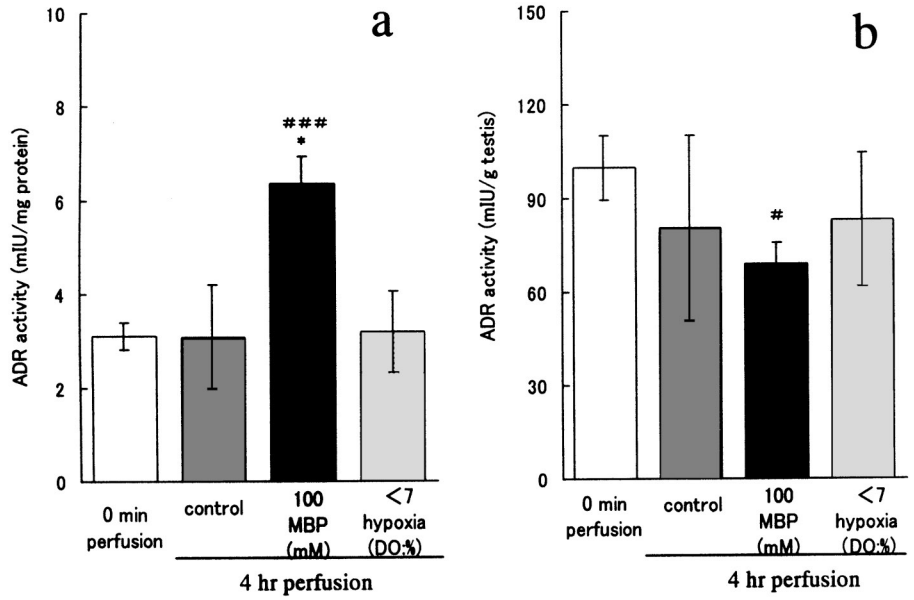

Fig. 6. ADR Activity in the Perfused Testis

a: activity per total protein, b: activity per g perfused testis. Values are mean ± S.D. for 5 rats. ###$p < 0.005$ compared with the 0 hr perfused control. *$p < 0.05$ compared with the 4 hr perfused control. DO is % of air (about 8.3 mg/l, 37°C). DO < 7% is corresponding to < 0.58 mg/l.

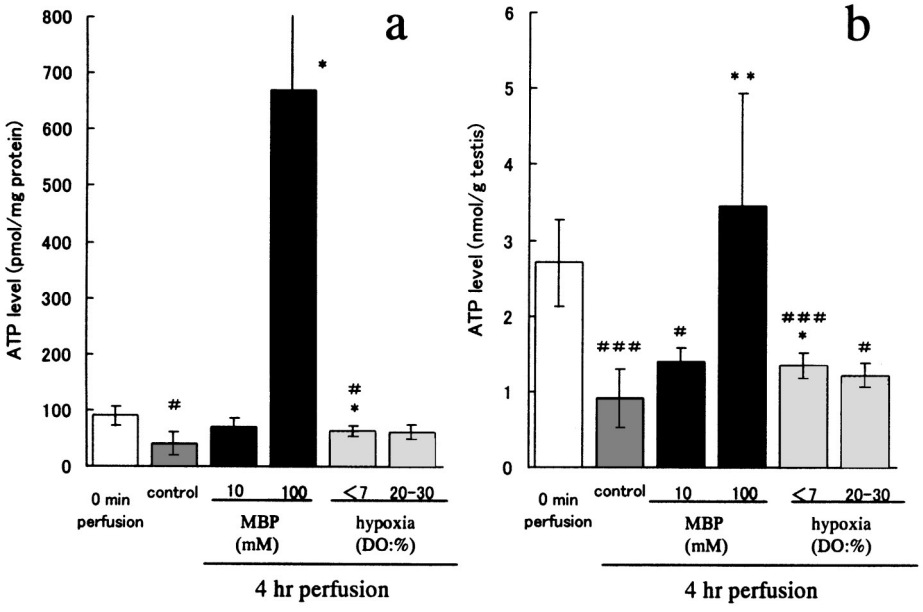

Fig. 7. ATP Level in the Perfused Testis

a: level per total protein, b: activity per g perfused testis. Values are mean ± S.D. for 3 or 5 rats. #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.005$ compared with the 0 hr perfused control. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ compared with the 4 hr perfused control. DO is % of air (about 8.3 mg/l, 37°C). DO < 7% is corresponding to < 0.58 mg/l and DO 20–30% is 1.66–2.49 mg/l.

caused iron depletion in the testis, along with higher levels of transferrin and the depletion of SUDH in Sertoli cells.[7,8] However, it is not established whether these phenomena were caused directly by MBP, a metabolite of DBP, or secondarily by hypoxia due to Hb deprivation induced by the metabolite. In an attempt to solve this, the testes were perfused with a solution of MBP in Eagle's MEM or in MEM with/without oxygen, and the toxic mechanisms were examined from the testicular enzyme activities.

In our previous *in vivo* study,[12] the MBP levels in the testis determined by eliminated testicular

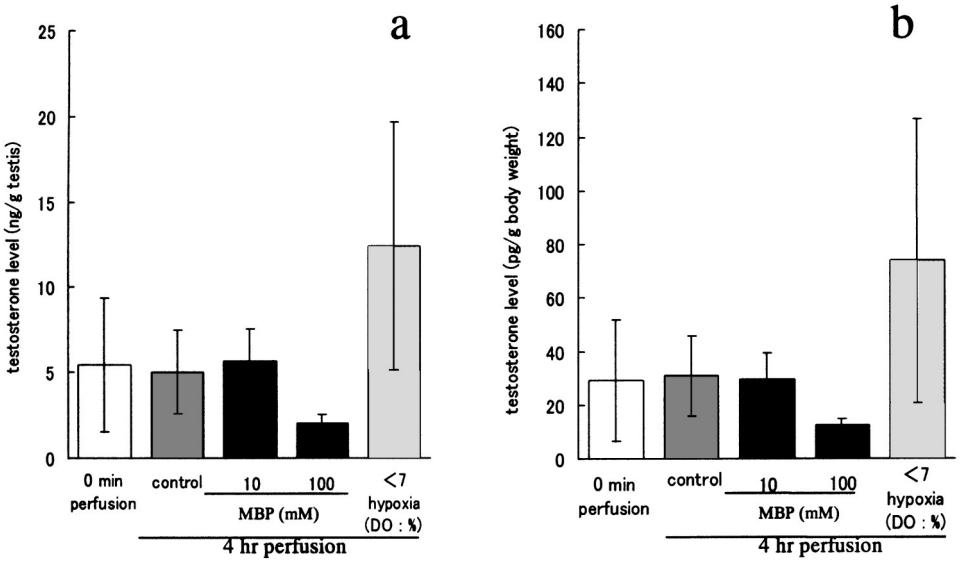

Fig. 8. Testosterone Level in the Perfused Testis

Values are mean ± S.D. for 5 rats and levels per g perfused testis. DO is % of air (about 8.3 mg/l, 37°C). DO < 7% is corresponding to < 0.58 mg/l.

blood, were observed at 55 ± 3.9 nmol/g testis at 1 hr, 114.4 ± 5.4 nmol at 3 hr, 60.8 ± 3.9 nmol (at 6 hr), 57.2 ± 1.1 nmol (at 12 hr) and 23.2 ± 1.1 nmol (at 24 hr), respectively, after orally toxic dosing of 8.6 mmol DBP/kg.[6–9,12] The maximal concentration in the testis was 10 mM MBP at 3 hr, corresponding to 1/10 (10%) concentration in the blood, considering that a total volume of the blood in rat testis was about 10 μl/g,[18] and that a partition coefficient of MBP was 0.12 at pH 7.4 and about 12% of MBP in the blood was anticipated to permeate through the blood-testis barrier into the Sertoli cells. The *in vivo* dosage induced the germ cell detachment from the Sertoli cells *in vivo*,[6–9,12] and indicated that the sum[12] of MBP concentrations among the Sertoli cell, Sertoli-germ connection and germ cell fractions was about 6 nmol; that is, the sum of 0.06 ± 0.04 nmol, 5.7 ± 0.6 nmol and 0.27 ± 0.04 nmol, respectively. The *in vivo* dosage corresponded to 62.5 amol/cell and 375 amol/cell from 6 nmol/Sertoli cells and 6 nmol/germ cells, respectively, considering that the total numbers[15] of the germ and Sertoli cells in rat testis were about means of 92×10^6 and 16×10^6 per rat testis, respectively. However, in the primary culture,[13,14] the germ cell detachment was observed at the micro M of MEHP, corresponding to 9.27 fmol/Sertoli cell. Accordingly, the toxic mechanism should be investigated using the *in vivo* dose levels because the *in vitro* doses[13,14] were a thousand times more than the *in vivo* toxic dose[6–9,12] (corresponding to 10 mM MBP in the testis and 100 mM in the perfusate).

When 10 mM and 100 mM MBP-perfusates were compared with the control ones (no existence of MBP), decreases in protein levels and enzyme activities were observed (Figs. 1, 2 and 6). The protein decrease at 100 mM MBP was supported by the *in vivo* results that a transfer of the activities in enzymes was observed from the Sertoli and germ cells to the seminiferous lumen at 3 to 6 hr after the toxic dosing of DBP.[7,12] Of the proteins, the activities/testis of SUDH and ADR showed decreases on the 100 mM MBP perfusion, but the activity of ALP increased. These results are consistent with previous *in vivo* results[12] that the activities of SUDH and ADR were lost from the Sertoli cells at 3 hr and 6 hr after the oral DBP dosing, respectively, and that the activity of ALP increased in the Sertoli cells and seminiferous lumen at 6 hr However, since the activities per protein level of the enzymes increased at 100 mM MBP, the study did not support the possibility that MBP directly caused a depletion of SUDH activity.

When the toxic dose of DBP was administered to rats orally, Hb levels were depleted to 83.9% and 84.1% at 3 and 6 hr, respectively, leading to a reduced oxygen transport capacity of the blood.[7] As for a hypoxic condition on the perfusion, it was difficult to prepare a stable perfusate with DO 50–90%, but a stable perfusate was attained at lower than 30% DO or higher than 95% DO. A hypoxic perfusate (20–30% DO) caused a decrease in SUDH activity, increases in ATP levels per both protein and testis,

Table 2. Body and Testis Weights and Testicular SUDH Activity of Rat Treated with DBP or 3-NPA

	BW (g)	testis weight (g) right (left)	testes/BW $\times 10^{-3}$	SUDH activity mIU/mg protein	SUDH activity mIU/testis
control	151.7 ± 2.9	0.878 ± 0.04 (0.891 ± 0.03)	11.8 ± 0.4	2.90 ± 0.3	112.8 ± 9.0
DBP	150.0 ± 5.0	0.753 ± 0.08 (0.767 ± 0.09)	10.1 ± 1.3	2.33 ± 0.2*	107.9 ± 17.2
3-NPA	150.0 ± 4.9	0.843 ± 0.01 (0.831 ± 0.03)	11.2 ± 0.2	2.04 ± 0.2*	087.8 ± 13.9

The results are means ± S.D. for 5 rats. BW means body weight. Enzyme activity in mIU g^{-1} testis. *$p < 0.05$ compared with the respective control; Dunnett-type mean rank test.[24)]

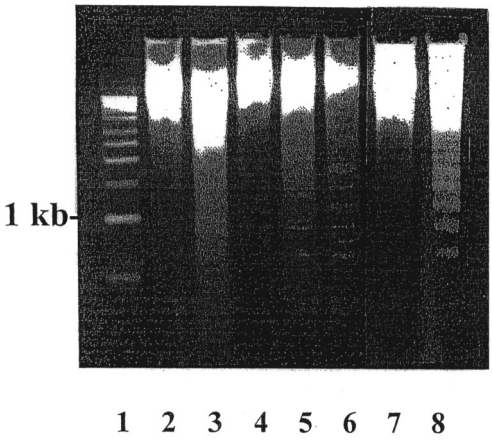

Fig. 9. Fragmentation of DNA Isolated from the Testis Perfused with 100 mM MBP or Low Oxygen Conditions, and the Testis of Rat Administered DBP or 3-NPA

Lane 1: DNA standard molecular markers, 2: 4 hr perfused control, 3: 100 mM MBP perfused testis, 4: 20–30% hypoxic condition, 5: lower than 7% hypoxic condition, 6: positive control,[23)] and 7 and 8: testes at 12 hr after dosing of 3-NPA and DBP, respectively.

an ascending tendency of testosterone and no change in either the activities of other enzymes (Figs. 2–6) or the levels of iron. The results supported a hypoxic mechanism relative to the SUDH depletion in the testes.

These findings related apoptosis, hypoxia and SUDH depletion (corresponding to ATP levels). Recently, Richburg and Boekelheide[16)] reported that MEHP exhibits apoptosis on dissociated germ cells. The Sertoli cells injured by MEHP stimulated an introduction of apoptosis on the germ cells, and this apoptosis might be associated with reducing the burden of Sertoli cells.[23,25)] In general, apoptosis in the testis played a significant role in normal development[26)] and mature spermatogenesis,[27)] as well as in experimental models such as ischemia[28)] and testicular toxicants.[29,30)] Hypoxia also induced apoptosis by activation of the p53 protein.[31)] Acute (short exposure) and chronic (long exposure) hypoxia induced decreases and increases in SUDH activities, respectively, of hepatic, renal and cerebral cells.[32,33)] However, it was unclear whether the apoptosis was attributed to Sertoli cell damage caused directly by MBP or secondarily by a hypoxia due to MBP-induced hemoglobin deprivation. In the perfused testis, apoptosis was observed under the hypoxic condition (lower than 7% DO), but not on the 100 mM MBP perfusion using DNA laddering on gel electrophoresis (Fig. 9). The hypoxic condition (lower than 7% DO) induced increases in ATP as did the 100 mM MBP perfusates (Fig. 7). Therefore, the relationship between ATP increase and apoptosis led to the hypotheses that testicular apoptosis might be induced by a hypoxic environment and that the ATP increase observed in the perfused testis was conceivable to be ATP unconsumed in or an agent in apoptotic germ cell death at lower than 7% DO or 100 mM MBP perfusion. The apoptosis was induced by ATP supply, or its partial depletion,[34)] whereas the necrosis was by complete ATP depletion.[34,35)] The ATP increase was observed in human neuroblast cells exposed early to 3-NPA, a SUDH inhibitor, and then the decrease was in the cells exposed longer, leading to delayed apoptosis.[21,22,36)] In this study, DBP induced depletion of SUDH activity and apoptosis, and 3-NPA induced SUDH depletion but not DNA ladders at 12 hr after the dosing, according to the method of Gould *et al.*[21)] Therefore, it appeared conceivable that SUDH depletion was followed by at least the induction of apoptosis in the testis. Moreover, the hypoxic condition (lower than 7% DO) induced an ascending tendency of testosterone content in the perfused testes (Fig. 8). This might be supported by the *in vivo* results that the Leydig cells of guinea pig produced an increase in the level of

testosterone by incubation under hypoxia (3% oxygen) culture conditions,[37] and that higher testosterone was found in the testes of rats receiving MBP orally.[10] A hypoxic environment (at 6000 m of high altitude) led to highly vacuolated spermatogenic tissues in male rats,[38] indicating testicular atrophy. Taking these observations together, MBP might contribute to an induction of the hypoxic state, followed by SUDH depletion and then apoptosis.

Accordingly, this study could support the possibility that testicular damage induced by phthalate might result from short-term hypoxia due to its metabolite. Expanding this possibility, it is conceivable that testicular atrophy results in the removal of apoptotic germ cells from the testicular tubule to conserve the species.

Taken together, this study proposes the possibility that MBP might cause hypoxia and/or a coincident depletion of SUDH activity followed by apoptosis in the testis.

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