HIF-1α or control antibody for 2 h at 4 °C. BpA or BpF was added to the cell lysate together with the antibody. Immunocomplexes were precipitated with protein G. The precipitates were subjected to SDS–polyacrylamide gel electrophoresis with 7.5% polyacrylamide gel. After Western blotting, Hsp90 or HIF-1α proteins were visualized using horseradish peroxidase conjugated to goat anti-rabbit IgG and by 4-chloro-1-naphthol.

## Results

### *Inhibition of EPO induction under hypoxia by BpA*

Hep3B cells were cultured under hypoxia. EPO mRNA was strongly induced, and addition of BpA completely suppressed the induction (Fig. 1A). HIF-1α mRNA levels did not change. However, HIF-1α protein levels were reduced by BpA. The inhibitory effect was increased by increase of BpA concentration, and the effects were dose-dependent (Fig. 1B). These results suggest that BpA promoted HIF-1α degradation under hypoxia following the inhibition of EPO induction.

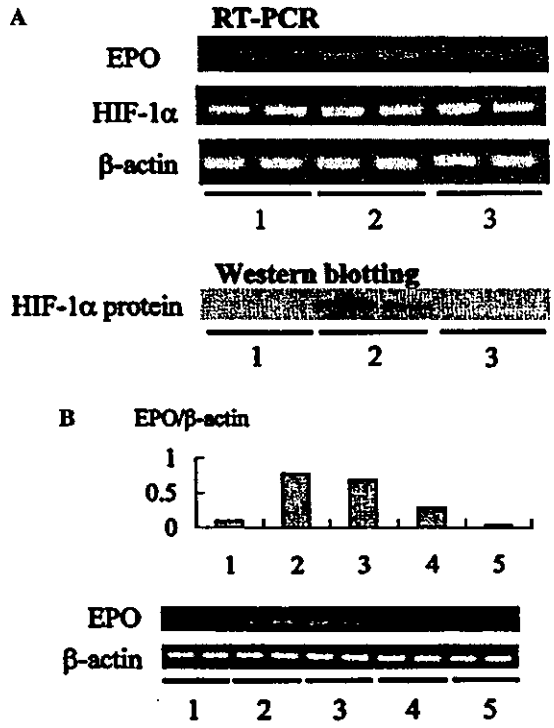


Fig. 1. Inhibition of EPO induction under hypoxia by BpA. (A) Hep3B cells were cultured under hypoxia for 6 h in the absence or presence of BpA (200 μM). Total RNA or protein was isolated from cells in two different culture plates, RT-PCR or immunoblotting was performed. 1, control (normoxia); 2, hypoxia; 3, hypoxia plus BpA. (B) Hep3B cells were cultured for 6 h in the presence of BpA (0, 50, 100, and 200 μM). The graph indicates the relative intensity of the EPO band normalized by β-actin. 1, control (normoxia); 2, hypoxia; and 3, hypoxia plus BpA (50 μM); 4, hypoxia plus BpA (100 μM); and 5, hypoxia plus BpA (200 μM).

### *Required chemical structure of BpA for inhibition of hypoxic response*

The structure of BpA, or 2,2-bis(4-hydroxyphenyl)-propane, consists of two parts, two phenols and propane (Fig. 2A). First, we blocked the hydroxyl groups of phenol by adding methyl groups, and this derivative was added to Hep3B cells under hypoxia (Fig. 2B). The resulting derivative had the same efficiency as the mother compound, BpA. Next, the methyl groups in the propane structure were removed one by one, resulting in bisphenol E (BpE) and bisphenol F (BpF). The inhibitory efficiency was increasingly reduced with the removal of each methyl group (Fig. 2C). These results suggest that the propane structure (two central methyl groups), but not the phenol structure in BpA, was important for the inhibition of EPO induction via the degradation of HIF-1α.

### *Inhibition of BpA on EPO induction by cobalt*

Cobalt ion mimics the hypoxic induction. Cobalt ion inhibits interaction of HIF-1α with pVHL, ubiquitin

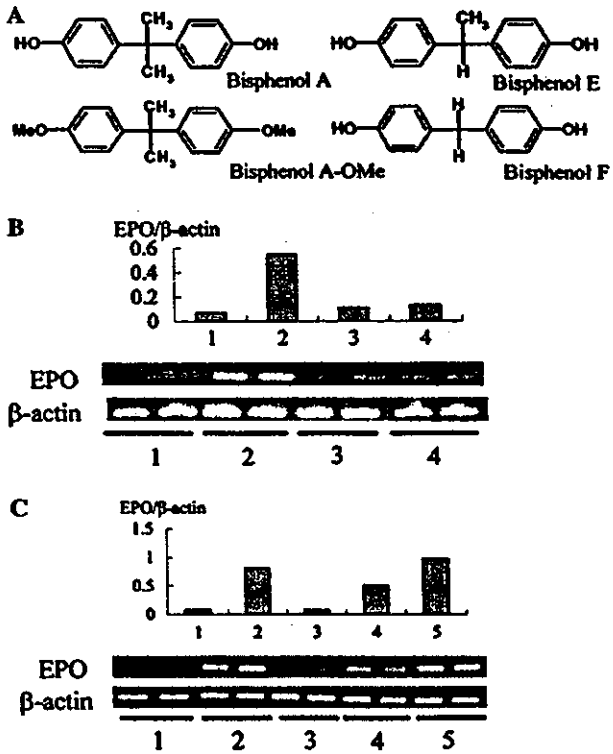


Fig. 2. Relation between BpA chemical structure and inhibition of EPO induction. (A) Chemical structures of BpA analogs. (B) Hep3B cells were cultured for 6 h under hypoxia in the presence of BpA (200 μM) or BpA-OMe (200 μM). 1, control (normoxia); 2, hypoxia; 3, hypoxia plus BpA; 4, hypoxia plus BpA-OMe. (C) Hep3B cells were cultured for 6 h under hypoxia in the presence of BpA, BpE, or BpF (each 200 μM). 1, control (normoxia); 2, hypoxia; 3, hypoxia plus BpA; 4, hypoxia plus BpE; and 5, hypoxia plus BpF.

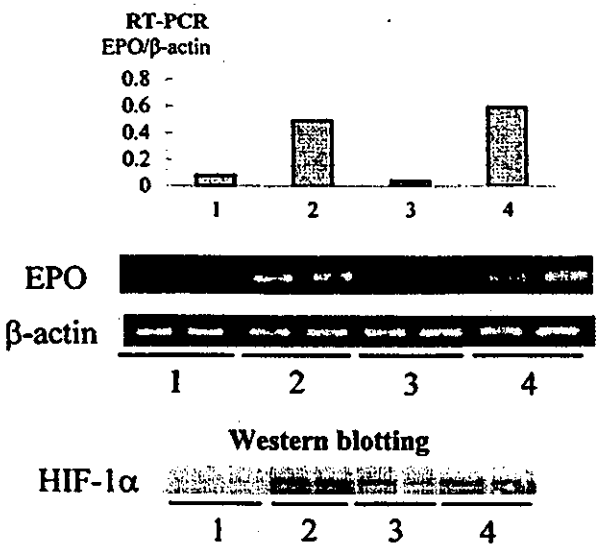


Fig. 3. Inhibition of EPO induction in the presence of cobalt ion by BpA. Hep B cells were cultured in the presence of cobalt ion (200 μM) for 6 h in the absence or presence of BpA (200 μM) or BpF (200 μM). Total RNA or protein was isolated from cells in two different culture plates, and RT-PCR or immunoblotting was performed. The graph indicates the relative intensity of the EPO band on an agarose gel after RT-PCR normalized by β-actin. Immunoblotting using homogenates of Hep3B cells was done with HIF-1α antibody. 1, control (normoxia); 2, treatment with cobalt ion under normoxia; 3, cobalt ion plus BpA; 4, cobalt ion plus BpF.

ligase for HIF-1α [12]. The inhibition produces accumulation of HIF-1α and hypoxic response proteins such as EPO are induced even under normoxia. This process occurs in spite of the prolyl hydroxylation of HIF-1α. BpA was added to Hep3B cells under normoxia with cobalt (Fig. 3). BpA completely inhibited EPO induction by cobalt as in hypoxia. BpF did not inhibit EPO induction, suggesting that the methyl groups in BpA were important for the inhibition. Immunoblotting indicated that BpA but BpF degraded HIF-1α protein in the presence of cobalt ion like in hypoxia. The inhibition pattern by cobalt treatment was the same as that by hypoxia. Hypoxia and cobalt ion stabilize HIF-1α by different mechanisms. Hypoxia inhibits proline hydroxylation in HIF-1α following ubiquitination by pVHL. Cobalt ion inhibits the interaction of HIF-1α with pVHL. BpA inhibited both pathways by inducing degradation of HIF-1α.

*Interaction of BpA with Hsp 90 and dissociation of HIF-1α from Hsp90*

Mabjeesh et al. [13] found that geldanamycin induces HIF-1α degradation independent of $O_2$ tension. Recently, it was reported that HIF-1α is associated with the chaperone protein Hsp90 [14]. Geldanamycin is a specific inhibitor for Hsp90. We therefore investigated whether BpA affects the interaction of HIF-1α with Hsp90 (Fig. 4). Hep 3B cells were cultured under

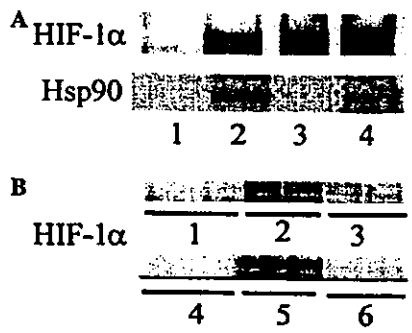


Fig. 4. Effects of BpA and BpF on immmunoprecipitation of HIF-1α and Hsp90 and on degradation of HIF-1α by proteasome. (A) Hep3B cells were cultured for 4 h under normoxia or hypoxia. The cells were homogenized. The homogenates were immunoprecipitated with HIF-1α or control antibodies in the absence or presence of BpA (200 μM) or BpF (200 μM). The precipitate was analyzed by immunoblotting with HIF-1α or Hsp90 antibodies. 1, control (normoxia); 2, hypoxia; 3 hypoxia plus BpA; 4, hypoxia plus BpF. (B) Hep3B cells were cultured under normoxia or hypoxia in the presence of a proteasome inhibitor, MG132 (10 μM). The cells from two different culture plates were homogenized and analyzed by immunoblotting with HIF-1α antibody. 1, control (normoxia); 2, treatment with MG 132 under normoxia; 3, treatment with MG132 and BpA under normoxia; 4 control (normoxia); 5, treatment with MG132 under hypoxia; and 6, treatment with MG132 and BpA under hypoxia.

hypoxia and homogenized. An antibody against HIF-1α or control serum was added to homogenates in the presence or absence of BpA. The resulting antibody complex was precipitated and analyzed by SDS–polyacrylamide gel electrophoresis following immunoblotting with an antibody against Hsp90. As previously reported, HIF-1α and Hsp90 were co-precipitated [14]. However, BpA inhibited the co-precipitation of HIF-1α and Hsp90, indicating that BpA inhibited the function of Hsp90 as in geldanamycin. Furthermore, BpF did not inhibit the co-precipitation, suggesting that the two methyl groups in BpA are required for the interaction of BpA with Hsp90. Moreover, MG132, a proteasome inhibitor, was added to Hep3B cells, and it increased the amount of HIF-1α protein under normoxia and hypoxia (Fig. 3B). The addition of BpA also decreased the amount of HIF-1α protein under both normoxia and hypoxia. These results suggest that the degradation of HIF-1α in Hep3B cells by treatment with BpA occurred via an as-yet-unidentified pathway rather than by the pVHL–proteasome pathway.

## Discussion

Bisphenol A (BpA) has physiological effects on reproductive, immunological, and nervous systems in mammals [15–17]. The effects of BpA on the reproducibility or feminization of animals have been extensively studied but the molecular mechanism of the immunological and nervous effects of BpA remains poorly

understood. In this study, we found that BpA inhibited the hypoxic response via the HIF-1 pathway of cells. Genetical deficiency of HIF-1α in mice results in developmental arrest, and the embryos manifest neural tube defects, suggesting that HIF-1α plays an important role in the development of embryos and the formation of neuronal systems [18]. Furthermore, prenatal and neonatal exposure to BpA in mice has been shown to enhance hyperlocomotion, concomitant with an increase of dopamine $D_1$ receptor mRNA in the whole brain [3]. In vivo, hypoxia decreases dopamine $D_1$ receptor levels [19]. It is not clear whether the expression of dopamine receptor is regulated by HIF-1, but it can be said that BpA had the opposite effect of hypoxia in this case. EPO is a typical hypoxia-inducible protein and is regulated by HIF-1. EPO is known as a hematopoietic cytokine and it induces erythroid differentiation. It has recently been reported that EPO acts to stimulate neural progenitor cells and to prevent apoptosis in the embryonic brain [11]. Together with these findings, our present results suggest that BpA causes abnormalities of the central neural system in the developmental stage by inhibiting HIF-1 function.

In this study, we found that BpA promoted HIF-1α degradation by dissociation of a chaperon protein, Hsp90, from HIF-1α. The antibiotic geldanamycin has the same effect, inhibiting Hsp90 function followed by HIF-1α degradation. However, the chemical structure of BpA is quite different from that of geldanamycin. Blocking of the phenol groups in BpA by methyl did not affect the inhibition activity or binding activity to Hsp90. However, the two methyl groups in the central position of BpA were important for the binding to Hsp90. Resveratrol, which is similar to BpA and has two phenol structures, also inhibited EPO induction, but it did not interact with Hsp90 (data not shown). Hsp90 recognized the BpA structure selectively. Furthermore, BpA inhibited EPO induction by cobalt, which stabilizes HIF-1α by inhibiting the interaction of HIF-1α with pVHL, a ubiquitin ligase. BpA also enhanced HIF-1α degradation in the presence of cobalt. Isaacs et al. [20] found that geldanamycin enhances HIF-1α degradation in pVHL-deficient cells and suggested that HIF-1α was degraded by a pVHL-independent pathway. BpA also seemed to degrade HIF-1α via a similar pathway. In addition, we found that BpA degraded HIF-1α in the presence of the proteasome inhibitor, MG132, which induced an accumulation of HIF-1α even under normoxia. These results suggest that BpA degraded HIF-1α via an unidentified pathway which is proteasome-independent.

## Acknowledgments

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# Apigenin suppresses the expression of VEGF, an important factor for angiogenesis, in endothelial cells via degradation of HIF-1α protein

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**Abstract Apigenin, a plant-derived flavone, is a potent inhibitor of cell proliferation and angiogenesis, but the mechanisms leading to the pathological anti-angiogenic effects of apigenin are still unclear. In this study, we found that apigenin inhibited the hypoxia-induced expression of vascular endothelial growth factor (VEGF) mRNA in human umbilical artery endothelial cells. Apigenin also suppressed the expression of erythropoietin mRNA, which is a typical hypoxia-inducible gene, via the degradation of hypoxia-inducible factor 1 (HIF-1) α. We investigated the effect of apigenin on the interaction of HIF-1α with heat shock protein 90 (Hsp90), which is reported to be important for the stabilization of HIF-1α, and found that VEGF expression was inhibited via degradation of HIF-1α through interference with the function of Hsp90.**


*Keywords:* Apigenin; Angiogenesis; Vascular endothelial growth factor; Hypoxia; Hypoxia-inducible factor; Heat shock protein 90

## 1. Introduction

Several polyphenolic compounds are recognized as cancer chemopreventive agents. Flavonoids are especially well known to suppress tumor cell growth via cell-cycle arrest and by the induction of apoptosis in several tumor cell lines [1–5]. Moreover, flavonoids inhibit endothelial cell proliferation and angiogenesis in vitro, the latter is endothelial cell cultures on collagen gels [6,7]. Angiogenesis is essential for the growth, progression, and metastasis of solid tumors [8]. Apigenin, a member of the flavone family that is present at high levels in many vegetables, has been found to inhibit ornithine decarboxylase and chemically induced skin tumorigenesis [9]. However, the mechanism by which apigenin suppresses angiogenesis has not been elucidated.

A prime regulator for angiogenesis is believed to be vascular endothelial growth factor (VEGF) and low oxygen tension dramatically induces the expression of this major angiogenic factor [10]. Transcriptional upregulation of VEGF has been implicated in the induction of genes; the induction is mediated by the specific binding of hypoxia-inducible factor 1 (HIF-1) to the hypoxia response element (HRE) [10–12]. The transcription factor HIF-1 is a heterodimer composed of HIF-1α and aryl hydrocarbon receptor nuclear translocator (ARNT), also known as HIF-1β [13,14]. Although the ARNT protein is readily found in cells, HIF-1α is virtually undetectable in normal conditions. When cells are subjected to hypoxic conditions, the protein levels of the HIF-1α subunit increase rapidly. The existence of HIF-1 suggests that in the presence of oxygen, HIF-1α is regulated by two separate mechanisms: one involving prolyl hydroxylase, which initiates the degradation of HIF-1α, and another involving asparagine hydroxylase, which inactivates the C-terminal transactivation domain of HIF-1α [15]. Under normoxic conditions, the proteasome-dependent degradation of HIF-1α is mediated by prolyl hydroxylation, which permits the binding of the von Hippel–Lindau protein (pVHL), a component of the E3 ubiquitin ligase [16,17]. Under hypoxic conditions, prolyl hydroxylation of HIF-1α is blocked and the transcription factor HIF-1 is stabilized. Detailed study of HIF-1α protein revealed a 200-amino-acid sequence called the oxygen-dependent degradation (ODD) domain [18]. This domain is responsible for the degradation of HIF-1α in the presence of oxygen. pVHL mediates the ubiquitination and degradation of HIF-1α by binding to the ODD domain under normoxic conditions. It has been well documented that cobalt, a transition metal, mimics the effects of hypoxia by stabilizing HIF-1α [19]. However, the biochemical mechanisms underlying this stabilization differ from those underlying the stabilization induced by hypoxia. We have already reported that HIF-1α signal transduction during hypoxia was mediated by NADPH-P450 reductase (NPR) [20]. But NPR has little or no effect on erythropoietin (EPO) mRNA induction by cobalt. A recent study demonstrated that cobalt inhibits HIF–pVHL interaction even after hydroxylation of the proline residue [21], leading to a rapid accumulation of HIF-1α protein.

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*Abbreviations:* VEGF, vascular endothelial growth factor; HIF-1, hypoxia-inducible factor 1; EPO, erythropoietin; pVHL, von Hippel–Lindau protein; Hsp90, heat shock protein 90; HRE, hypoxia response element; ARNT, aryl hydrocarbon receptor nuclear translocator; ODD, oxygen-dependent degradation; NPR, NADPH-P450 reductase; HUA-EC, human umbilical artery endothelial cells; RT-PCR, reverse-transcription PCR; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; PAS, Per-ARNT-Sim; bHLH, basic helix loop helix

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In this study, we tested the hypothesis that the anti-angiogenic effect of apigenin on tumor cells is caused by a reduction in the expression of VEGF, which is regulated by HIF-1 under hypoxic conditions. Moreover, we investigated a mechanism by which apigenin induces degradation of HIF-1α, independent of the ubiquitination of pVHL.

## 2. Materials and methods

### 2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), EGM-2 medium, fetal calf serum, and *N*-CBZ-Leu-Leu-Norvalinal (proteasome inhibitor) were purchased from Sigma Chemical Co. (St. Louis, MO). Isogen was obtained from Nippon Gene (Toyama, Japan). An RNA PCR Kit (AMV) Ver. 2.1 was purchased from Takara (Shiga, Japan). Anti-human HIF-1α IgG and anti-human ARNT IgG were obtained from Novus (Littleton, CO) and Abcam (Cambridgeshire, UK), respectively. Anti-human heat shock protein 90 (Hsp90) IgG was purchased from BD Biosciences (San Jose, CA). Protein G-Sepharose 4 Faster Flow was supplied by Amersham Biosciences Corp. (Piscataway, NJ). Nitrocellulose membrane and 4-chloro-1-naphthol were purchased from Bio-Rad Laboratories (Hercules, CA). The Vectastain ABC kit (a biotin/avidin system) was obtained from Vector Laboratories (Burlingame, CA).

### 2.2. Cell culture

The human hepatoma cell line Hep3B was obtained from the Cell Resource Center for Biomedical Research at the Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). Human umbilical artery endothelial cells (HUA-EC) were obtained from Cambrex Bioscience Bio-Whittaker (Walkersville, MD). The Hep3B cells were maintained in DMEM containing 10% fetal calf serum and the HUA-EC in EGM-2 containing 10% fetal calf serum. To stimulate hypoxia, the cells were incubated in 5% $O_2$, 5% $CO_2$ and 90% $N_2$ balanced with a modulator incubator chamber (Napco 7101, Winchester, VA) or in a sealed 2.5-L box with an Anero Pack (oxygen absorber) for cells (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan).

### 2.3. Isolation of RNA and reverse-transcription PCR (RT-PCR)

With the use of Isogen, total RNA was extracted from Hep3B cells and from HUA-EC. The total RNA was transcribed into cDNA using the RNA PCR Kit according to the manufacturer's protocol. PCR with 10 pmol of each primer, 1.5 U of Ampli Taq, and 100 ng of cDNA was performed for 10 min at 94 °C and then 35 cycles of 1 min at 94 °C, 1 min at 56 °C and 2.5 min at 72 °C. The PCR primers for EPO [22] were 5′-GCCAGAGGAACTGACCAGAG-3′ (sense) and 5′-TTCTTCAGGTCATCCTATCC-3′ (antisense), while the PCR primers for VEGF [23] were 5′-TTCATGGATGTCTATCAGCG-3′ (sense) and 5′-CATCTCTCCTATGTGCTGGC-3′ (antisense). The oligonucleotide sequences of the reaction products were confirmed by sequencing.

### 2.4. Western blotting and immunoprecipitation

Cells at 80% confluence were lysed in 20 mM Tris–HCl buffer (pH 8.0) containing 0.1% Tween 20, 10% glycerol, 5 mM $MgCl_2$, 300 mM KCl, and 20 μM *N*-CBZ-Leu-Leu-Norvalinal. Cell lysates were divided into two and incubated with 2.8 μg of anti-HIF-1α IgG at 4 °C. Apigenin (100 μM) was added together with anti-HIF-1α IgG. After 2 h, 60 μL of protein G (10% vol/vol in lysis buffer) was added to each incubation mixture, and the incubation continued for another 2 h. Samples were washed twice by lysis buffer and protein G was removed by centrifugation. Samples were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) with 7.5% polyacrylamide gel. Using a previously described method [24], we performed immunoblotting with anti-ARNT IgG, anti-Hsp90 IgG, or antibodies against HIF-1α that were prepared in our laboratory [20]. Each protein was visualized using the ABC kit and 4-chloro-1-naphthol.

## 3. Results

### 3.1. Effect of apigenin on VEGF expression under hypoxia

HUA-EC were used to investigate the inhibitory effect of apigenin on VEGF expression (Fig. 1). When HUA-EC were exposed to 5% $O_2$ for 6 h, VEGF mRNA expression increased markedly. However, VEGF mRNA disappeared completely when apigenin (20 μM) was added to the culture medium. These results show that the flavone apigenin inhibited VEGF mRNA expression under hypoxic conditions.

### 3.2. Effect of apigenin on EPO expression during hypoxia

To examine whether or not apigenin inhibits the expression of EPO mRNA induced by hypoxia, we used human hepatocyte (Hep3B) cells and analyzed EPO mRNA expression by RT-PCR. The Hep3B cells were exposed to 5% $O_2$ for 3 h and then the expression of EPO mRNA was observed under hypoxic conditions (Fig. 2A). Apigenin dose-dependently reduced the expression. As HIF-1 was the transcription factor of the EPO gene during hypoxia, we studied the levels of HIF-1α and ARNT in Hep3B cells under hypoxic conditions by Western blotting (Fig. 2B). Although no immunoreactive band for HIF-1α was observed under normoxic conditions in Hep3B cells, HIF-1α was clearly expressed during hypoxia. When apigenin (100 μM) was added to the culture medium, the expression of HIF-1α was reduced. On the other hand, the levels of ARNT, a subunit of HIF-1, were not at all affected by treatment with apigenin.

### 3.3. Effect of apigenin on EPO expression induced by cobalt

Cobalt is known to inhibit the interaction between HIF-1α and pVHL directly and thereby prevent the degradation of HIF-1α [21]. To examine whether or not apigenin inhibits the cobalt-induced stabilization of HIF-1α, Hep3B cells were treated with apigenin in the presence of cobalt (Fig. 3A). Apigenin inhibited the expression of EPO mRNA induced by cobalt. Moreover, we tested the effect of apigenin on the levels of HIF-1α in Hep3B cells treated with cobalt (Fig. 3B). HIF-1α expression increased on treatment with cobalt, whereas it apparently decreased when apigenin (100 μM) was added to the culture medium. These results indicate that apigenin inhibits the stabilization of HIF-1α in the presence of cobalt. The presence of apigenin did not change the ARNT levels. When apigenin was present, HIF-1α was degraded under hypoxic conditions or in the presence of cobalt ion under normoxic conditions, suggesting that apigenin degraded HIF-1α via a pVHL-independent pathway.

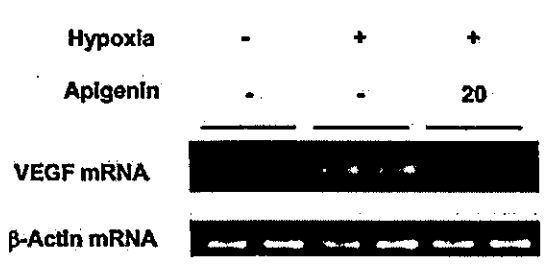


Fig. 1. Expression of VEGF mRNA in HUA-EC. HUA-EC were exposed to 5% $O_2$, 5% $CO_2$ and 90% $N_2$ for 6 h in the presence or absence of 20 μM apigenin. VEGF mRNA expression was analyzed by RT-PCR.

### 3.4. Hsp90 is required for the stabilization of HIF-1α during hypoxia

Using geldanamycin, a specific inhibitor of Hsp90, Minet et al. [25] demonstrated that Hsp90 was essential for stabilizing HIF-1. In addition, HIF-1α belongs to the Per-ARNT-Sim (PAS)-basic helix loop helix (bHLH) family and interacts with Hsp90 [25,26]. We hypothesized that the degradation of HIF-1α by apigenin correlates with the interaction between HIF-1α and Hsp90. We prepared cell lysates from Hep3B under conditions of hypoxia in the absence of apigenin. Apigenin was added to the lysates together with anti-HIF-1α IgG and then the complex anti-HIF-1α IgG was collected using protein G. The samples were analyzed by SDS–PAGE with 7.5% polyacrylamide gel. The antibody against HIF-1α could pull down Hsp90 protein, indicating that Hsp90 is associated with HIF-1α (Fig. 4). When apigenin was added to the cell lysates together with the antibody, the band of Hsp90 disappeared, indicating that apigenin inhibited the interaction of HIF-1α with Hsp90. Geldanamycin also reduced the levels of EPO mRNA in Hep3B cells under hypoxic conditions (data not shown).

## 4. Discussion

Dietary factors contribute to about one-third of potentially preventable cancers, and the preventive effects of plant-based diets on tumorigenesis and other chronic diseases have been well documented [27]. Several cancers, including breast cancer, have a lower incidence in Asia than in Western countries. This has been attributed to the Asian dietary regimen, which is typically rich in flavonoid-containing plants. Researchers have identified the isoflavonoid genistein as a potent inhibitor of angiogenesis in vitro [6,7]. A recent study demonstrated that the anti-angiogenic effect of genistein was due to inhibition of HIF-1, an important regulator of VEGF gene homeostasis particularly under low-oxygen conditions [28]. The flavone apigenin has also been proposed as an antitumor agent [29]. However, the molecular mechanisms involved in the anti-angiogenic effects of apigenin are not well understood. This study investigated the anti-angiogenic effects of apigenin on cells under hypoxic conditions. We observed that apigenin suppressed the expression of both VEGF mRNA and EPO mRNA induced by hypoxia in HUA-EC and in Hep3B cells. Apigenin proved more effective against the expression of VEGF mRNA and EPO mRNA than genistein (data not shown). In this study, we found that apigenin caused the degradation of HIF-1α but not of ARNT. Given that genistein has been reported to inhibit HIF-1 DNA-binding activity [28], apigenin and genistein may have different angiogenic mechanisms.

Apigenin inhibited the mRNA expression of EPO induced by cobalt, which mimics hypoxia; it also inhibited the stabilization of HIF-1α induced by cobalt. Because the mechanism underlying the stabilization of HIF-1α during hypoxia is known to differ from that induced by cobalt, we speculate that the degradation of HIF-1α by apigenin is independent of the ubiquitination by pVHL. As geldanamycin reduced the expression of EPO mRNA in Hep3B cells under hypoxic conditions (data not shown), we examined the effect of apigenin on the interaction with HIF-1α of the chaperone protein Hsp90, which associates with HIF-1α to stabilize an activated form of HIF-1 during hypoxia [25,30]. Through immunoprecipitation experiments, we found that apigenin disturbed the binding of Hsp90 to HIF-1α. These results indicate that apigenin diminishes the hypoxia-induced interaction of HIF-1α with Hsp90 to release HIF-1α, which in turn undergoes degradation.

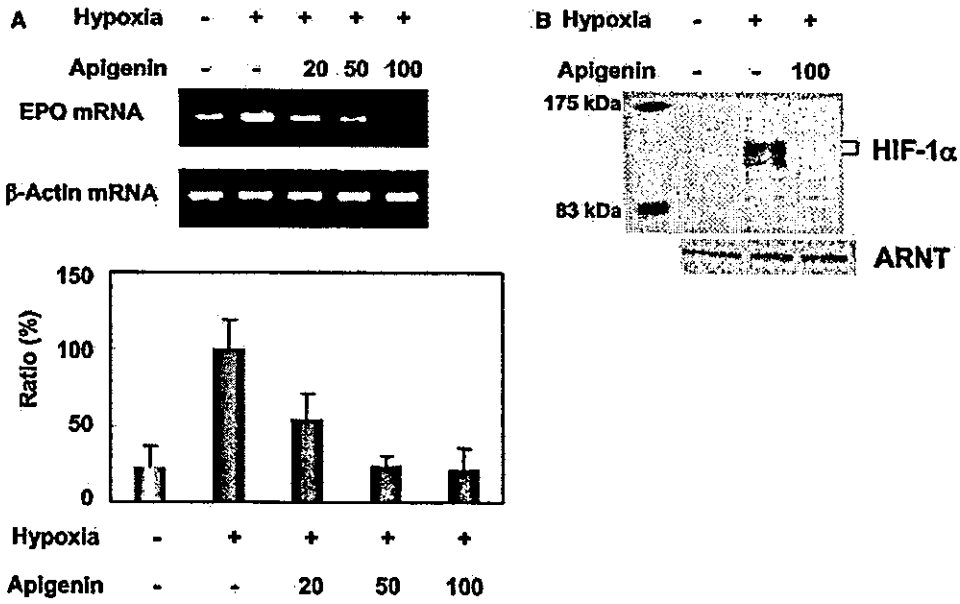


Fig. 2. Expression of EPO mRNA and HIF-1α in Hep3B cells under hypoxic conditions. (A) Hep3B cells were exposed to 5% $O_2$, 5% $CO_2$ and 90% $N_2$ for 3 h in the presence or absence of 20, 50, or 100 μM apigenin. EPO mRNA expression was analyzed by RT-PCR. The ratio (EPO mRNA/β-Actin mRNA) under hypoxia was 100%. Values are given as means ± S.D. for four separate experiments. (B) Hep3B cells were exposed to 5% $O_2$, 5% $CO_2$ and 90% $N_2$ for 3 hours in the presence or absence of 100 μM apigenin. Whole cell lysates were immunoprecipitated with anti-HIF-1α IgG and analyzed by SDS–PAGE with 7.5% poly-acrylamide gel, and then were immunoblotted with antibodies against HIF-1α. Whole cell lysates (50 μg) were analyzed by SDS–PAGE with 7.5% polyacrylamide gel and immunoblotted with anti-ARNT IgG. An ABC kit was used for the secondary antibody and HIF-1α was detected with 4-chloro-1-naphthol.

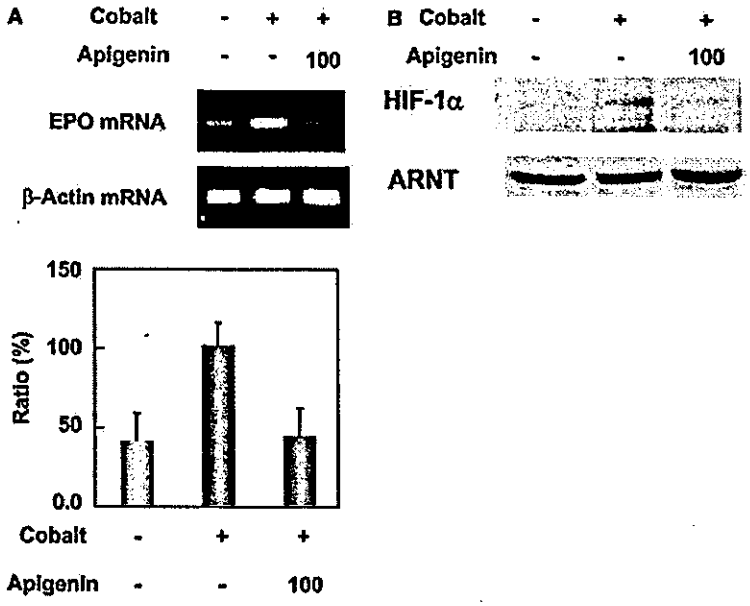


Fig. 3. Expression of EPO mRNA and HIF-1α in Hep3B cells treated with cobalt. Hep3B cells were exposed to 100 μM cobalt for 3 h in the presence or absence of 100 μM apigenin. (A) EPO mRNA expression was analyzed by RT-PCR. The ratio (EPO mRNA/β-Actin mRNA) under hypoxia was 100%. Values are given as means ± S.D. for four separate experiments. (B) Whole cell lysates were analyzed by SDS–PAGE with 7.5% poly-acrylamide gel and immunoblotted with antibodies against HIF-1α (1:100). An ABC kit was used for the secondary antibody and HIF-1α was detected with 4-chloro-1-naphthol.

We studied whether or not flavonoids other than apigenin have an inhibitory effect on the expression of EPO mRNA induced by hypoxia. We found that kaempferol and myricetin have an inhibitory effect on the expression under hypoxic conditions, but catechin and epicatechin do not (data not shown). Wilson et al. [31] demonstrated that the flavonoid quercetin stabilizes HIF-1α and induces the expression of VEGF mRNA under normoxic conditions. Moreover, it has been proposed that quercetin regulates the response to hypoxia by inhibiting the activity of HIF hydroxylase, a member of the Fe(II), 2-oxoglutarate-dependent dioxygenase family, because quercetin is a good iron chelator [32].

In this study, we found that apigenin reduced HIF-1α levels by interfering with the binding of HIF-1α to Hsp90, leading to a suppression of EPO mRNA transcription. The angiogenesis frequently observed in tumors would be suppressed by apigenin via a reduction of VEGF mRNA expression. Since it may suppress a number of genes induced by HIF-1α, it is likely that apigenin could be a potent inhibitor of angiogenesis. Our results clearly show that apigenin is potentially an important chemical for controlling HIF-1α levels.

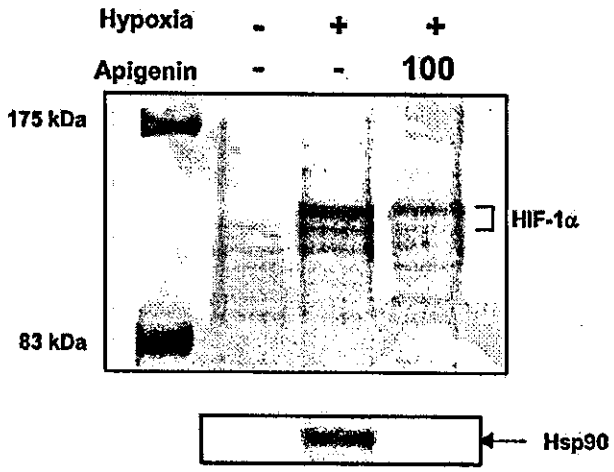


Fig. 4. Interaction of HIF-1α and Hsp90 proteins in the presence of apigenin. Hep3B cells were exposed to 5% $O_2$, 5% $CO_2$ and 90% $N_2$ for 3 h. Apigenin (100 μM) was added to whole cell lysates. The lysates were immunoprecipitated with anti-HIF-1α IgG and analyzed by SDS–PAGE with 7.5% polyacrylamide gel and immunoblotted with antibodies against HIF-1α (1:100) (A) and anti-Hsp90 IgG (B).

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with the two antiandrogens. Rat testicular protein extracts of control and treated animals were analysed by 2-D gel electrophoresis coupled with MALDI-TOF. This technique can detect differentially expressed proteins between control and treated testicular samples. Of the total protein population observed, we identified a number of proteins which are involved in several cellular processes: detoxification mechanism, apoptosis, translation, metabolism, signaling pathway, immune response, chaperones. Further investigations will be necessary to find the relevant protein which ultimately contribute to the cellular changes observed in the testis following treatment with antiandrogens.

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## EFFECT OF BISPHENOL A ON CENTRAL NERVOUS SYSTEMS

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Bisphenol A (BPA) is a monomer of polycarbonate plastics and used in the lining of food cans and dental sealants. BPA is suspected to be one of endocrine disrupting chemicals (EDCs) due to its acting as an agonist for the estrogen receptors. Although many investigations have suggested that some chemicals disrupt reproductive systems, the effects on central nervous systems are not well known. We previously found that prenatal and lactational exposure of BPA reduced dopamine (DA) contents in mice. Since BPA was suspected to affect central nervous system through dopaminergic neuron, we convinced that the novel receptor of BPA presented in synaptosomal membrane fraction in the brain. In this study, we isolated and purified the BPA binding protein from rat synaptosomes using BPA-Sepharose affinity column chromatography technique. N-terminal sequence of the purified protein was identical with protein disulfide isomerase (PDI) which was also known as thyroid hormone binding protein. The competition binding assay was performed to elucidate possible molecular mechanism of the BPA action on thyroid hormone binding. BPA inhibited the binding of 3, 3', 5-triiodothyronine ($T_3$) to PDI with an $IC_{50} = 22.1$ μM which was 10-100 fold lower affinity than $T_3$ ($IC_{50} = 0.79$ μM). This effective potency of BPA suggest interfere the thyroid hormone action via PDI. We also examined the displacing activity of $T_3$ from PDI by other chemicals which were suspected as EDCs. The binding of $T_3$ to PDI was inhibited by 4-octylphenol, 4-nonylphenol, pentachlorophenol, 2, 4-dichlorophenol, tetrabromobisphenol A, and tetrachlorobisphenol A. BPA and these chemicals mimic the thyroid hormone action by competing with thyroid hormone bound to PDI.

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## ENDOCRINE DISRUPTING CHEMICALS - OLD AND NEW - REASONS FOR CONTINUED PUBLIC HEALTH INTERVENTION

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The United Nations Environmental Programme indicates the production of new chemicals are increasing every year on a global scale. There are approximately 50,000 to 100,000 chemicals being produced commercially. The potential human health threat from exposure to these chemicals is unknown. Chemicals such as polybrominated diphenyls ethers (PBDEs), perfluorosulfonates (PFOS), chlorinated naphthalenes, perchlorates and others are new threats to the environment and human health. All of these chemicals have been found in the environment, and have been shown to bioaccumulate in humans. For example, in the U.S. Great Lakes basin PBDEs have been found in air, sediment, biota, wildlife, human fatty tissue, blood serum, and breast milk. The chemical structure of PBDEs is similar to PCBs and therefore may cause neurological effects from exposure. Initial animal studies indicate PBDEs can cause neurologic effects from exposure. Perchlorates are colorless and ordorless salts used in explosives and rocket motors and persist in water and soil. Perchlorates affect the ability of the thyroid gland to make hormones that regulate many body functions and development. This presentation will discuss these new emerging chemicals of concern and provide new information about health effects from the old persistent organic pollutants (POPs), such as polychlorinated biphenyls.

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## SPECIES DIFFERENCES IN THE EXPRESSION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORα (PPARα)-RELATED ENZYMES BY DI (2ETHYLHEXYL) PHTHALATE (DEHP)

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Male CD-1 mice, SD rats and marmosets (*Callithrix jacchus*) were treated with 0, 1.25 and 2.5 mmol/kg DEHP (for mice and rats) for two weeks, and with 0, 0.25, 1.25 and 6.25 mmol/kg DEHP (for marmosets) for fifteen months by gavage. On the day after the last dose (18-20 hours later), all animals were killed by $CO_2$ asphyxiation. The induction of PPARa-related peroxisomal and mitochondrial enzymes were investigated.

Immunoblotting analysis clearly showed a band detected by anti-CYP4A in the liver from rats, but not in the livers of mice or marmosets. The PT and PH detected by anti-PT and anti-PH in the livers from mice and rats, but not in livers from marmosets, are induced by exposure to DEHP. These enzyme inductions were most prominent in livers from rats, followed by mice. MCAD and VLCAD detected by anti-MCAD, and anti-VLCAD were also induced by DEHP exposure in mice and rats, but to a lesser extent compared with those in the peroxisomal enzymes. In contrast, these enzymes could not be induced in the liver from marmosets.

MCAD-mRNA levels were induced in the livers from mice and rats exposed to DEHP, but that from marmosets tended to decrease after exposed to 1.25 and 6.25 mmol/kg DEHP. PT-mRNA levels were strongly induced in the liver from rats exposed to DEHP, following by levels from mice, whereas those of marmosets decreased after exposure to 1.25 or 6.25 mmol/kg in the same manner as MCAD. These results are quite similar to changes in the respective proteins. Thus, we concluded that there are also species differences in DEHP-induced PPARα-related enzymes; species differences in induction of peroxisomal enzymes are most prominent.

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## A STUDY ON THE DEVELOPMENTAL TOXICITY OF ESTROGENIC CHEMICALS IN EARLY LIFE STAGE OF FISH

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The endocrine system plays an essential role in the development, growth, reproduction, and behavior of human beings and animals. A balance of steroid hormones in organism is essential to maintain normal gonadal differentiation and morphological development. Some environmental chemicals have been reported to possess the hormone-like activities. Thus they may result in reproductive disturbances and affect the developmental process.

The purpose of this study was to determine the effects of nonylphenol, bisphenol A and diethylstilbestrol (DES), which were known to have estrogenic activity, on the morphological development of Japanese med-

J Toxicol Pathol 2004; 17: 177–185

Original

# Di-n-butyl Phthalate is Toxic to the Male Reproductive System and Its Toxicity is Enhanced by Thioacetamide Induced Liver Injury

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**Abstract:** The modifying effects of liver injury on male reproductive organ toxicity of di-n-butyl phthalate (DBP) were studied using male F344 rats. Seventy-two male animals, aged 10 weeks at the commencement, were divided into 8 groups of 9 rats each. Groups 1 to 4 were given 200 mg/kg bw of thioacetamide (TAA) intraperitoneally 3 times / week, while Groups 5 to 8 were injected with the PBS vehicle only. From 1 week after the start, groups 1 and 5, 2 and 6, and 3 and 7 were given daily 500, 125, 31.25 mg/kg bw of DBP, respectively (23 times) for 4 weeks, while groups 4 and 8 served as vehicle controls. As the result of our treatment, liver injury was induced in the animals, and the liver injury was confirmed by histopathological findings and serum transaminase levels at sacrifice. Enhancement of rat male reproductive organ toxicity of DBP under liver injury status was also seen. The relative organ weights of prostate, bilateral seminal vesicles, bilateral epididymides and bilateral testes, and the sperm number and motility rate were decreased. Also the sperm abnormality rate was increased under liver injury. Furthermore histopathological abnormality of the testis, such as absence of germ cells and the presence of Sertoli cells only was seen in the rats treated with TAA and high dose of DBP, however neither the serum nor testicular testosterone levels varied in the present study. The sperm number and relative weight of bilateral epididymides were decreased by TAA treatment only, unrelated to DBP treatment. These facts suggest that liver injury can not only enhance the rat male reproductive organ toxicity of DBP, but also induce such toxicity by itself. (J Toxicol Pathol 2004; 17: 177–185)

**Key words:** DBP, male reproductive organ toxicity, F344 rat, TAA, liver injury

## Introduction

Recently, there has been increasing concern about the effects of environmental chemicals on human reproduction. A large number of chemical agents have been reported to affect reproduction in laboratory animals and some of them have been demonstrated to also influence humans[1]. For example, di-n-butyl phtalate (DBP), a widely used industrial solvent and plasticizer, has been recognized as a ubiquitous contaminant of the environment and is suspected to be an endocrine disrupter[2]. Blount *et al.*[3,4] noted several phthalate ester metabolites at the ppm level in the urine of people in the general population and found that mono-n-butyl phthalate, the major active metabolite of DBP, demonstrated its highest concentrations in women of childbearing age in the 20-40 year age group. They did not report exposure levels resulting in these urinary concentrations, but others[5] have used the data and estimated with reasonable assumptions that for the average female, the maximum exposure to DBP via the oral route would be ~113 μg/kg/day. This value is more than two orders of magnitude lower than the NOAEL (50 mg/kg/day) for DBP from animal studies[6,7]. In Japan, however, food contamination with DBP contained in plastic gloves has recently become a cause for concern.

DBP has been shown to exert hepatotoxic, cytotoxic, mutagenic effects and produce renal, pulmonary and reproductive dysfunction in experimental animals[8]. It is doubtful whether the liver would be a target of this chemical in humans, because the effects are due to species specific actions on peroxisomes[9]. Much attention, however, has been paid to its male reproductive organ toxicity. At doses ≥ 1 g/kg bw/day, DBP is a testicular toxicant in rodents, causing seminiferous tubule degeneration, with young animals being more susceptible than adults to this toxic effect[10]. At lower dose levels (≥ 250 mg/kg/day), when administered during the major period of male reproductive organogenesis in the

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rat (gestation days 12–21), DBP blocks male reproductive development, resulting in feminization of male pups, malformation of the male reproductive organs, seminiferous tubule degeneration, and Leydig cell hyperplasia and adenomas[11].

DBP is hydrolyzed by lipase to mono-n-butyl phthalate, which is thought to be the active form[12]. This material is then oxidized in the liver and excreted into the urine in experimental animals[12]. It is thought that the mechanism of testicular atrophy induced by DBP in rats might be associated with iron release from hemoglobin and/or transferrin in the liver[13]. Liver dysfunction might delay the metabolism of DBP and thus enhance its male organ reproductive toxicity, although there have hitherto been no relevant experimental data in support of this supposition. In the present study, therefore, the modifying effects of liver injury on male reproductive organ toxicity of DBP were studied using F344 rats.

Thioacetamide (TAA) is a thiono-sulfur-containing compound which is widely used as an organic solvent in the leather, textile and paper industries, and as an accelerator in the vulcanization of rubber and as a stabilizer of motor fuel. It also causes liver damage and hepatoneoplasia. Oren R *et al.*[14] reported that oral administration of TAA (0.03% in tap water) for 12 weeks induced severe distortion of the liver architecture with generation of nodules surrounded by fibrotic septa in livers of female Wistar rats. Similar results were obtained in a study using male F344 rats in our laboratory (unpublished data). Furthermore, Reif S. *et al.*[15] reported that liver cirrhosis was induced in male Wistar rats by intraperitoneal injection of TAA, 200 mg/kg twice weekly for 12 weeks. After establishment of a suitable protocol to induce liver injury by TAA, we here investigated its modifying effects on the male reproductive organ toxicity of DBP in F344 rats in the present study.

## Materials and Methods

### *Animals*

Seventy-two male F344/DuCrj rats were obtained at 9 weeks of age from Charles River Japan Inc. (Hino, Shiga). They were housed 3 animals per stainless steel cage with paper chips for bedding and placed in a controlled environment, air conditioned at 24 ± 2°C, with a relative humidity of 55 ± 20% and a 12h/12h light-dark cycle. Food (CE-2 pellet diet; Clea Japan Inc., Tokyo) and water were available *ad libitum* throughout the period of the experiment. Experimentation was started after 1 week of acclimation and was approved by the Institutional Animal Care and Use Committee of Osaka City University Medical School.

### *Chemicals*

DBP was purchased from Tokyo Kasei Co. Ltd. (Tokyo) and TAA from Sigma Chemical Co. (St. Louis, MO, USA). Medium 199 with Hank's salt and L-glutamine was obtained from Gibco Co. (NY, USA) and 1.0% w/v bovine serum albumin from Intergen Co. (NY, USA). Corn oil was purchased from Wako Pure Chemical Industries Ltd. (Osaka). All chemicals used were of the highest purity commercially available.

### *Experimental design*

Srivastava *et al.*[16] reported that DBP treatment of Wistar albino rats caused decrease of the sperm count and disorganization of seminiferous tubules at a dose of 500 mg/kg body weight, but no marked histopathological changes at 250 mg/kg body weight after 15 days of oral administration. Therefore the high dose of DBP used in our study was set as 500 mg/kg body weight. At 10 weeks of age, the rats were divided into 8 groups (9 rats each) (Fig. 1). Animals in groups 1 to 4 were given 200 mg/kg body weight of TAA with 3 ml of PBS from the commencement of the experiment to week 5 (ip., 3 times a week). Groups 5 to 8 were injected with 3 ml of PBS only as vehicle controls. From 1 week after the start of the experiment, groups 1 and 5 were given 500 mg/kg body weight of DBP daily with 3 ml of corn oil (ig. 5 times a week). Groups 2 and 6 similarly received 125 mg/kg body weight and groups 3 and 7 were administered 31.25 mg/kg body weight. Groups 4 and 8 were given corn oil only. The doses of TAA used in the present study were chosen based on the results of a preliminary study (unpublished data). Body weights were measured every other day during week 1 and every day during weeks 2 to 5. Food consumption and water intake were measured once weekly. All rats were sacrificed under ether anesthesia at week 5, and liver, kidneys, prostate (ventral, dorsolateral), seminal vesicles with coagulate glands, testes and epididymides were excised and weighed. Before sacrifice, blood samples were taken for measurement of serum transaminase and testosterone levels, and frozen samples of left testes and epididymides were also taken and used for measurement of testosterone levels by radioimmunoassay.

### *Tissue processing and morphological examination*

The right testis and epididymis of each animal were fixed in GFA (glutaraldehide, formalin and acetic acid) solution for 1 day and replaced in 10% phosphate-buffered formalin solution. Slices 2–3 mm thick were taken from three lobes of livers and fixed with samples of kidneys and prostate fixed in 10% phospate-buffered formalin. The tissues were routinely processed for embedding in paraffin, sectioned at 3 μm and stained with hematoxylin and eosin (H and E) for histopathological examination. Liver sections were also processed for Azan and Mallory (A and M) staining.

### *Sperm analysis*

Six rats from each group were used for sperm analysis.

Sperm counts: The left epididymis resected at sacrifice were homogenized and sonicated with 10 ml water, and sperm heads were counted using a hemocytometer under a light microscope and recorded. The densities were calculated on the basis of the dilution rate.

Sperm motility tests: One droplet of sperm was

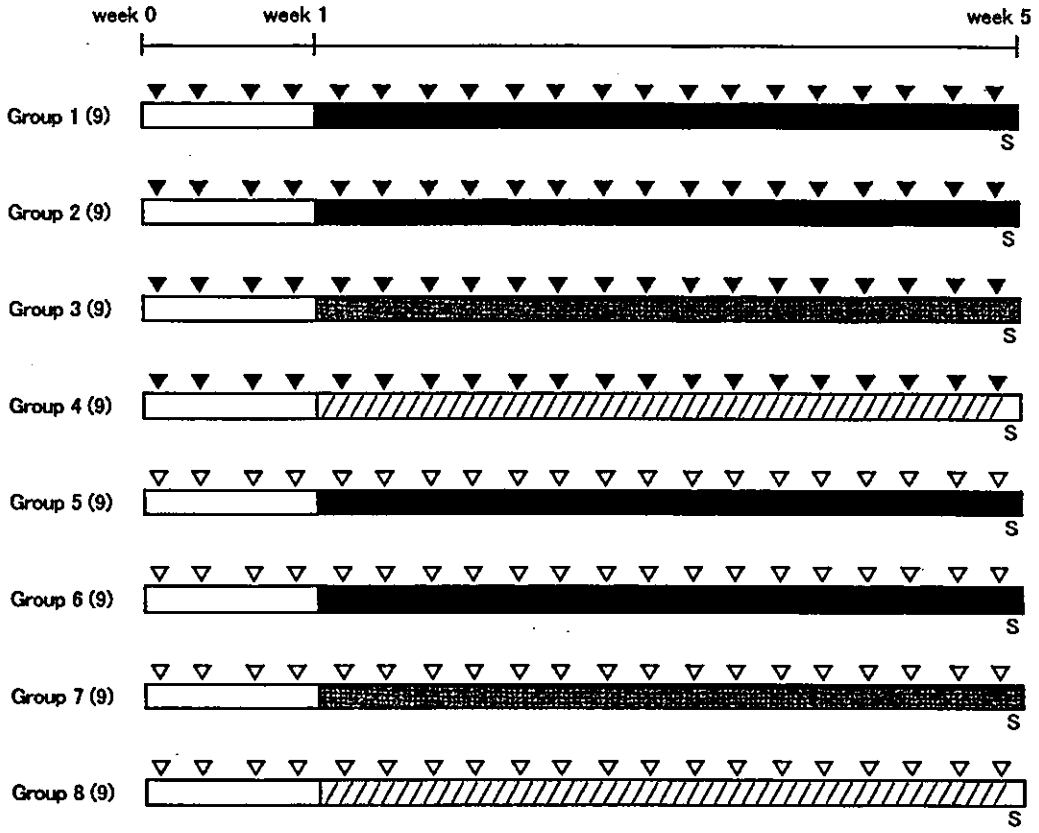


**Fig. 1.** The experimental protocol. Animals were 10-week-old male Fischer 344 rats. ▼; TAA, 200 mg/kg. Ip 3 times per week. ▽; vehicle (PBS) only ip 3 times per week. Black, Dark gray and Light gray; DBP at 500, 125 and 31.25 mg/kg/day ig. with 0.4 ml of corn oil once a day for 28 days. Cross hatched; vehicle (corn oil) only ig. once a day for 28 days. S; time points of sacrifice. Number of animals examined is shown in parentheses.

collected from a puncture of the left caudal epididymis, and introduced into 2 ml of sperm suspension medium [Medium 199 with Hank's salt and L-glutamine (Gibco Co. NY, USA)] supplemented with 1.0% w/v bovine serum albumin (Intergen Co. NY, USA) maintained at 37°C. The sperm suspensions were plated at 37°C for 5 min so that sperm could diffuse completely in the media, then examined under an optical microscope and the numbers of motile sperm were counted for a total of 100 sperm. The % motility was calculated from the data using the following formula: number of motile sperm × 100 / total number of observed sperm.

Sperm morphology: The suspensions for sperm motility analysis were also used for counts of abnormal sperm in 200 sperm. The samples were dried on hot plate and fixed for observation. Abnormal shapes were defined as follows: no head, no hook, banana shape head, excessive hook, pin head, two head, amorphous, two tails and short tail.

*Statistical analysis*

Statistical analyses were performed with Stat-View software for Macintosh computers. Student's *t* test was used for testing differences in body weights, relative organ weights, food consumption, water intake, sperm numbers, sperm motility rates, sperm abnormality rates, serum transaminase levels and serum and testicular testosterone levels between liver injury induced rats and rat without liver injury. For testing the dose effect of DBP as significance, the post hoc test was used. Values of $p<0.05$ and $<0.01$ were considered as significant difference.

## Results

*Body weight, water intake and food consumption*

Growth retardation in groups 1 to 4 was observed, compared to the corresponding groups of rats without liver injury induction, groups 5 to 8 (Table 1), and was also slight but significant in group 5 compared to group 8. Furthermore, food consumption was significantly less than in each of the corresponding control groups (groups 5 to 8) (Table 1). In contrast, water consumption was similar in all groups (Table 1).

*Relative organ weights*

The relative weights of liver, bilateral kidneys, seminal

**Table 1.** Body Weight, Water Intake and Food Consumption

| Group | Treatment | | Examined No. of animals | Body weight (g) | | Food consumption (g/day/rat) week 1–4 | Water intake (ml/day/rat) week 1–4 |
|---|---|---|---|---|---|---|---|
| | TAA | DBP | | Initial | Final | | |
| 1 | + | 500 mg/kg/day ig. | 9 | 224 ± 5 | 206 ± 7[a] | 8.9[a] | 23 |
| 2 | + | 125 mg/kg/day ig. | 9 | 222 ± 6 | 211 ± 11[a] | 8.4[a] | 20 |
| 3 | + | 31.25 mg/kg/day ig. | 9 | 223 ± 5 | 207 ± 10[a] | 8.4[a] | 21 |
| 4 | + | corn oil only | 9 | 224 ± 5 | 206 ± 16[a] | 9.5[a] | 22 |
| 5 | – | 500 mg/kg/day ig. | 9 | 222 ± 6 | 270 ± 13[b] | 14 | 22 |
| 6 | – | 125 mg/kg/day ig. | 9 | 223 ± 3 | 276 ± 9 | 14 | 22 |
| 7 | – | 31.25 mg/kg/day ig. | 9 | 223 ± 7 | 278 ± 9 | 14 | 21 |
| 8 | – | corn oil only | 9 | 223 ± 6 | 285 ± 9 | 15 | 21 |

Data are mean ± SD values.
ig.; intragastrial injection. a; Significantly different from each corresponding non-TAA treatment groups (group 5 to 8), $p < 0.05$.
b; Significantly different from non-TAA treatment and corn oil only ig. group. (group 8), $p < 0.05$.

**Table 2.** Relative Organ Weight of Animals

| Group | Treatment | | Examined No. of animals | Liver | Kidneys | Testes | Epididymides | Prostate | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | TAA | DBP | | | | | | ventral lobe | dorsolateral lobe | seminal vesicles + coagulate glands |
| | | | | (%) | (%) | (%) | (%) | (%) | (%) | (%) |
| 1 | + | 500 mg/kg/day ig. | 9 | 4.97 ± 0.28 | 0.85 ± 0.08[a] | 0.39 ± 0.04[a] | 0.18 ± 0.03[a,b] | 0.06 ± 0.03[d] | 0.04 ± 0.02[a] | 0.06 ± 0.02[d] |
| 2 | + | 125 mg/kg/day ig. | 9 | 4.77 ± 0.22[a] | 0.82 ± 0.05[a] | 1.06 ± 0.13 | 0.28 ± 0.03 | 0.06 ± 0.03[d] | 0.07 ± 0.03[d] | 0.07 ± 0.02[d] |
| 3 | + | 31.25 mg/kg/day ig. | 9 | 5.03 ± 0.40[a] | 0.83 ± 0.06[a] | 1.11 ± 0.08[a] | 0.30 ± 0.04 | 0.06 ± 0.02[d] | 0.05 ± 0.02[d] | 0.07 ± 0.01[d] |
| 4 | + | corn oil only | 9 | 4.85 ± 0.57[a] | 0.82 ± 0.08[a] | 1.08 ± 0.11 | 0.28 ± 0.04 | 0.08 ± 0.04[d] | 0.06 ± 0.02[a] | 0.07 ± 0.01[d] |
| 5 | – | 500 mg/kg/day ig. | 9 | 4.40 ± 0.23[c] | 0.71 ± 0.03[c] | 1.01 ± 0.07 | 0.30 ± 0.02 | 0.14 ± 0.04 | 0.09 ± 0.04 | 0.28 ± 0.05 |
| 6 | – | 125 mg/kg/day ig. | 9 | 3.72 ± 0.23[c] | 0.66 ± 0.02[c] | 0.99 ± 0.13 | 0.29 ± 0.03 | 0.15 ± 0.02 | 0.11 ± 0.03 | 0.29 ± 0.02 |
| 7 | – | 31.25 mg/kg/day ig. | 9 | 3.50 ± 0.12 | 0.64 ± 0.02 | 1.05 ± 0.04 | 0.30 ± 0.02 | 0.14 ± 0.02 | 0.11 ± 0.02 | 0.29 ± 0.04 |
| 8 | – | corn oil only | 9 | 3.33 ± 0.07 | 0.63 ± 0.02 | 1.02 ± 0.10 | 0.39 ± 0.02 | 0.15 ± 0.03 | 0.11 ± 0.05 | 0.31 ± 0.02 |

Data are mean ± SD values.
ig.; intragastrial injection. a; Significantly different from each corresponding non-TAA treatment groups (group 5 to 8), $p < 0.05$. b; Significantly different from TAA treatment and corn oil only ig. group (group 4), $p < 0.05$. c; Significantly different from non-TAA treatment and corn oil only ig. group (group 8), $p < 0.05$. d; Significantly different from each corresponding non-TAA treatment group. (group 5 to 8), $p < 0.01$.

vesicles with coagulate glands in groups 1 to 4 were significantly larger than those of each corresponding control group (groups 5 to 8), except for the liver value in group 1 (Table 2). The relative weights of bilateral epididymides in group 1 were significantly lower than in group 4, and the relative weights of the bilateral testes of groups 1 and 3 were significantly lower than those of the corresponding groups of rats without liver injury induction, groups 5 and 7 (Table 2).

*The results of sperm analysis*

The results of sperm analysis are summarized in Table 3. Sperm numbers of groups 1 to 4 were significantly lower than those of each corresponding control group (groups 5 to 8), and the sperm number of group 1 was also significantly lower than that of group 4 (Table 3). Furthermore, the sperm number of group 6 was significantly lower than that of group 8 (Table 3). The sperm motility rate for group 1 was also significantly lower than those for both groups 4 and 5 (Table 3).

Furthermore, sperm abnormality rates of groups 2 to 4 were significantly larger than those of each corresponding control group (6 to 8) (Table 3), and those of groups 5 to 7 were also significantly higher than that of group 8 (Table 3).

*Serum transaminase levels*

Both serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were significantly elevated in groups 1 to 4 compared to each corresponding non-TAA treatment group (5 to 8) (Table 4). However, no differences in serum transaminase levels were apparent between any of the liver injury-induced groups (groups 1 to 4) and their counterparts without liver injury induction (groups 5 to 8).

*Serum and tissue testosterone levels*

There was no specific variation in either serum or tissue testosterone levels in TAA or DBP treated animals (Table 5).

*Histopathological findings*

On histopathological examination, fibrosis, increased

**Table 3.** The Data of Sperm Analyses of Animals

| Group | Treatment | | Examined No. of animals | Sperm number (/ml) | Sperm motility rate (%) | Sperm abnormality rate (%) |
|---|---|---|---|---|---|---|
| | TAA | DBP | | | | |
| | | | | $\times 100^2$ | | |
| 1 | + | 500 mg/kg/day ig. | 9 | $45 \pm 70^{a,b}$ | $2.9 \pm 4.7^{a,b}$ | $23.2 \pm 10.9$ |
| 2 | + | 125 mg/kg/day ig. | 9 | $1083 \pm 368^{a}$ | $57.6 \pm 30.1$ | $30.0 \pm 9.8^{a}$ |
| 3 | + | 31.25 mg/kg/day ig. | 9 | $1245 \pm 373^{a}$ | $75.4 \pm 20.3$ | $20.9 \pm 10.4^{a}$ |
| 4 | + | corn oil only | 9 | $1452 \pm 533^{a}$ | $78.5 \pm 23.3$ | $29.1 \pm 10.4^{a}$ |
| 5 | – | 500 mg/kg/day ig. | 9 | $2748 \pm 683$ | $82.4 \pm 5.8$ | $17.7 \pm 7.8^{c}$ |
| 6 | – | 125 mg/kg/day ig. | 9 | $2067 \pm 287^{c}$ | $85.1 \pm 9.1$ | $16.4 \pm 2.6^{c}$ |
| 7 | – | 31.25 mg/kg/day ig. | 9 | $3093 \pm 713$ | $86.1 \pm 6.4$ | $9.3 \pm 4.3^{c}$ |
| 8 | – | corn oil only | 9 | $3172 \pm 479$ | $74.2 \pm 36.6$ | $2.4 \pm 1.1$ |

Data are mean ± SD values.
ig.; intragastrial injection. a; Significantly different from each corresponding non-TAA treatment groups (group 5 to 8), $p < 0.05$. b; Significantly different from TAA treatment and corn oil only ig. group (group 4), $p < 0.05$. c; Significantly different from non-TAA treatment and corn oil only ig. group (group 8), $p < 0.05$.

**Table 4.** Serum Transaminase Levels of Animals

| Group | Treatment | | Examined No. of animals | Serum transaminase level (IU/L/37°C) | |
|---|---|---|---|---|---|
| | TAA | DBP | | AST | ALT |
| 1 | + | 500 mg/kg/day ig. | 9 | $258 \pm 89^{a}$ | $136 \pm 43^{a,b}$ |
| 2 | + | 125 mg/kg/day ig. | 9 | $191 \pm 47^{a}$ | $90 \pm 17^{a}$ |
| 3 | + | 31.25 mg/kg/day ig. | 9 | $132 \pm 18^{a}$ | $64 \pm 12^{a}$ |
| 4 | + | corn oil only | 9 | $192 \pm 63^{a}$ | $88 \pm 30^{a}$ |
| 5 | – | 500 mg/kg/day ig. | 9 | $84 \pm 10$ | $41 \pm 5$ |
| 6 | – | 125 mg/kg/day ig. | 9 | $75 \pm 10$ | $42 \pm 4$ |
| 7 | – | 31.25 mg/kg/day ig. | 9 | $77 \pm 6$ | $47 \pm 4$ |
| 8 | – | corn oil only | 9 | $84 \pm 10$ | $45 \pm 4$ |

Data are mean ± SD values.
ig.; intragastrial injection. AST; aspartate aminotransferase. ALT; alanine aminotransferase. a; Significantly different from each corresponding non-TAA treatment groups (group 5 to 8), $p < 0.05$. b; Significantly different from TAA treatment and corn oil only ig. group (group 4), $p < 0.05$.

**Table 5.** Serum and Testicular Testosterone Levels of Animals

| Group | Treatment | | Examined No. of animals | Serum Testosterone Level (ng/ml) | Examined No. of animals | Testicular Testosterone Level (ng/ml) |
|---|---|---|---|---|---|---|
| | TAA | DBP | | | | |
| 1 | + | 500 mg/kg/day ig. | 9 | $0.74 \pm 0.78$ | 5 | $22.8 \pm 11.5$ |
| 2 | + | 125 mg/kg/day ig. | 7 | $1.29 \pm 1.51$ | 5 | $42.7 \pm 31.5$ |
| 3 | + | 31.25 mg/kg/day ig. | 7 | $1.62 \pm 1.76$ | 4 | $37.1 \pm 25.2$ |
| 4 | + | corn oil only | 9 | $1.09 \pm 1.43$ | 5 | $13.5 \pm 10.8$ |
| 5 | – | 500 mg/kg/day ig. | 8 | $1.71 \pm 1.70$ | 5 | $29.1 \pm 16.5$ |
| 6 | – | 125 mg/kg/day ig. | 9 | $3.16 \pm 4.62$ | 5 | $24.9 \pm 12.2$ |
| 7 | – | 31.25 mg/kg/day ig. | 9 | $1.61 \pm 1.21$ | 5 | $24.9 \pm 10.7$ |
| 8 | – | corn oil only | 9 | $1.36 \pm 1.39$ | 5 | $11.7 \pm 4.2$ |

Data are mean ± SD values.
ig.; intragastrial injection.

area of Glisson's sheath, pigmentation and infiltration of inflammatory cells were seen in the liver samples from groups 1 to 4 (Figs. 2A, 2C). There was no such evidence of liver injury in groups 5 to 8 (Figs. 2B, 2D).

In the testis and epididymis, findings pointing to male reproductive organ toxicity were seen only in group 1; seminiferous tubules showed severe atrophic changes and the normal architecture was distorted and disorganized (Fig. 3A), and some epithelial cells of the ducts in the epididymis showed cells shed from the seminiferous tubules in the

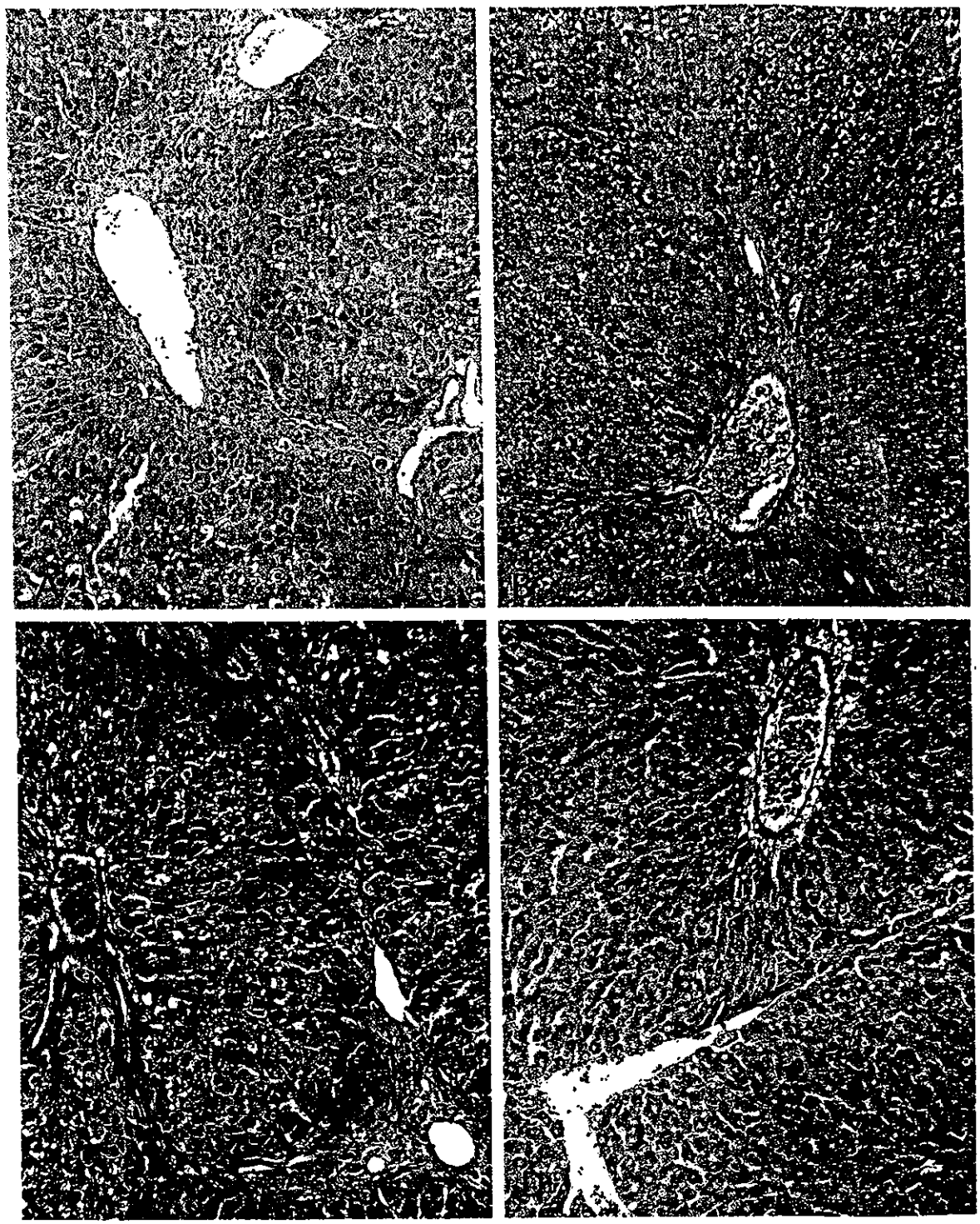

**Fig. 2.** Histopathological features of liver sections. Note fibrosis, increased area of Glisson's sheath, infiltration of inflammatory cells and pigmentation in a liver sample from group 1, in contrast to the control case (group 8). (A, B; H-E staining, × 200. C, D; A-M staining, × 200.).

lumina (Fig. 3C). Atrophic changes in testis were very mild in groups 2 to 7, and testes of group 8 demonstrated normal spermatogenesis (Fig. 3B) with no specific alteration evident in the epididymis (Fig. 3D).

Atrophic changes were apparent in prostate samples of liver injury-induced rats, but not in those of rats without liver injury induction.

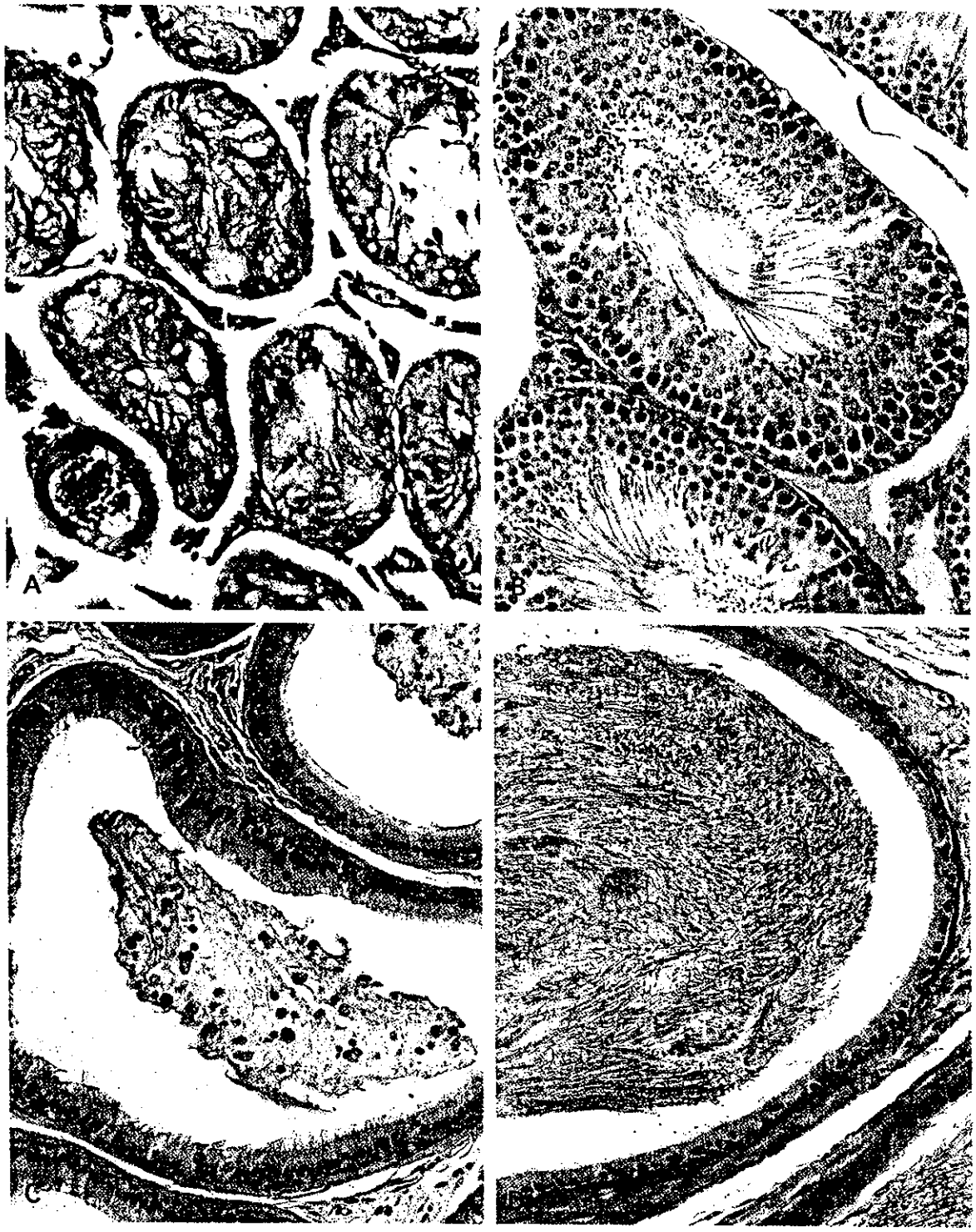


**Fig. 3.** Histopathological examinations of testis and epidydimis sections. Note evidence of male reproductive organ toxicity in the TAA treatment and DBP high dose group (group 1); seminiferous tubules show severe atrophic changes and the normal architecture is disturbed (A; H-E staining, × 200.), some of the epithelial cells of the ducts in an epididymis showing cells shed from seminiferous tubules evident in the lumen (B; H-E staining, × 200.). A testis of the non-treatment group 8 shows normal spermatogenesis (C; H-E staining, × 200.) and no specific alteration in the epididymis (D; H-E staining, × 200.).

## Discussion

The present study clearly showed that liver injury induced by TAA enhances the toxicity of DBP in the male sexual organs. Furthermore, weak but significant rat male reproductive organ toxicity could be seen in the sperm numbers and sperm abnormality rate in TAA -administered rats without DBP. Whether this is direct or indirect remains to be clarified.

Male reproductive organ toxicity of DBP was limited to increased sperm abnormalities in rats without liver injury induction, whereas the sperm number, motility rate and relative organ weights of testes and epididymis were all altered in liver injury-induced rats.

The sperm number of 125 mg/kg/day DBP-treated rats was significantly low compared to that of control rats. Although this result is more sensitive to the toxicity of DBP than the results of our histopathological examination, there was no dose-response. Thus, it was considered to be incidental.

The relative liver weights of the 500 and 125 mg/kg/day DBP-treated rats were significantly higher than those of control rats, and this was probably due to the hepatotoxic effect of DBP.

The relative kidney weights of the 500 and 125 mg/kg/day DBP-treated rats were significantly higher than those of control rats, and the relative kidney weights of the liver injury-induced rats were also significantly higher than those of rats without liver injury induction. These findings were probably due to the relative low body weights of the treated animals.

In the present study, liver injury induced by TAA showed some level of rat male reproductive toxicity including sperm counts and sperm abnormality rate, suggesting that TAA might modify hepatic clearance of steroid hormones via liver injury. It is indeed well known that patients with advanced liver disease show testicular atrophy and morphological and morphometric changes in alcoholic and nonalcoholic liver disease in humans have been described by several authors[17–20]. A rat model for alchohol-induced gonadal atrophy has also been reported[21]. In fact, hepatic cirrhosis is one of the classical conditions known to have a profound adverse effect on male reproductive function[22], and the combination of testicular failure and features of feminization is well-known[23–26]. Hypogonadism and feminization are not confined to alcoholic cirrhosis and may accompany other types of cirrhosis[23,24], and milder forms may occur with non-cirrhotic liver disease[23]. It would thus appear that the TAA influence is likely to be indirect via its effects on liver function. However, the hypothesis that reduced hepatic clearance of estrogen results in elevated blood levels and secondary suppression of gonadotropin secretion and testicular function has been disproved[24,25], and it is now recognized that there is mainly a primary testicular disorder with elevated gonadtropin levels and reduced testosterone production[24].

It has previously been reported that phthalate esters show no direct effect on the prostate[2], however, atrophic changes of this organ were seen in our animals, and it is assumed that this finding was a result of the liver injury.

It has been not clarified how DBP induces male reproductive organ toxicity in animals, although anti-androgenic effects have been demonstrated in animal studies using both sexes of rats[2]. Mylchreest E *et al.*[12] reported that DBP treatment caused a marked decrease in testosterone levels in the fetal testes of Sprague-Dawley rats.

Unexpectedly, there was no significant difference of the serum and testicular testosterone levels in our rats, though apparent anti-androgenic findings were observed. The small sample number might be the cause of this contradiction.

The sperm number in the epididymis of rats treated with both TAA and 125 or 31.5 mg/kg of DBP were diminished, but the relative epididymis weights of these rats were not diminished. We can offer no explanation for this apparent contradiction.

It has been reported that mono-n-butyl phthalate, the major metabolite of DBP, disrupts Sertoli cell-germ cell interactions *in vitro*[26,27]. Furthermore, Mylchreest E *et al.*[12] reported that DBP treatment did not induce atrophy of the prostate, the major target organ of flutamide, a well-known androgen receptor antagonist. Gray *et al.*[28] also reported the lack of interaction of DBP and mono-n-butyl phthalate with androgen receptors *in vitro*. Thus, Koizumi M *et al.*[2] concluded that the mechanism of action of DBP is not via androgen receptors. Estrogenic effects of DBP *in vitro* have been described[29,30], but Koizumi M *et al.*[2] could not confirm these *in vivo*. Further examination is needed to clarify the mechanisms underlying the results of the present study.

In conclusion, the results of the present study suggest that liver injury enhances the rat male reproductive organ toxicity of DBP, and this phenomenon might be caused by the inhibition of its metabolism in the liver and its accumulation. Furthermore, this implies that other endocrine disrupters which are detoxified in the liver will be also having their effects enhanced under liver dysfunction. The effects of DBP on humans should not be underestimated and further examination of this issue is needed.

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