と考えられるが、児動物への持続的な曝露に関する情報が不足している場合には児への直接投与について考慮する必要がある。持続曝露の証拠は、薬物動態に関する情報、児毒性、生体指標の変化から得られる。

#### 4.3.5. 母動物の観察

少なくとも1日1回、母体の健康状態を観察する。

妊娠中及び授乳中には少なくとも2回、1群当たり少なくとも10母体について詳細な状態を観察する。動物の観察は熟練した観察者が盲検でケージ外において動物のストレス及び観察者の先入観を最小にして標準的な手順により行う。可能であれば、試験を通じて同一の観察者により観察を行う。

皮膚、被毛、眼、粘膜、分泌、自律神経作用（流涙、瞳孔の大きさ、異常呼吸/口呼吸、排尿/排便の異常）等の状態観察を行う。姿勢、活動（探索の減少または増加）、行動の協調性等に関する異常反応を記録する。歩行の変化、姿勢の変化、取り扱い時の反応、環境刺激に対する反応、間代性または強直性の動作、痙攣、振戦、常同行動（過剰なグルーミング、頭部の異常な動き、反復旋回等）、異常行動（噛みつきないし過剰な舐め、自傷行動、後退り、異常発声等）、攻撃行動を記録する。

毒性兆候の発見日、時間、強さ及び持続時間を記録する。

試験期間を通じて少なくとも週1回、分娩日、離乳日（生後21日）に母体重を測定する。強制経口投与実験では少なくとも週2回母体重を測定する。投与量は体重に基づいて算出する。摂餌量は妊娠中及び授乳中に少なくとも週1回測定する。飲水投与の場合には少なくとも週1回飲水量を測定する。

#### 4.3.6. 児動物の観察

全ての児動物の状態を少なくとも1日1回観察する。少なくとも各母体の雌雄各1匹について、投与期間、観察期間中に、母動物の観察と同様に詳細に観察する。観察項目は発生の時期に応じ適宜選定する。児動物の身体発育及び機能／行動検査の時期を表5に示した。

体重は身体発育の最も良い指標であり、身体発達指標（耳介展開、開眼、切歯萌出等）の変化は、体重によく相関する。

身体発育の評価に際しては生後日齢の代わりに交尾後の日数を使うことが望ましいことがある。離乳日の児動物の検査は離乳のストレスを避けるために離乳の前に実施する。離乳直後の2日間における児動物の検査は避ける。

生存児については数、性別を記録し、分娩直後、授乳中に少なくとも週1回個別に体重を測定する。性成熟の検査として、少なくとも各母体当たり雌雄各1匹について、膣開口または包皮分離の完了日齢及び体重を記録する。

正向反射、背地走性、運動活性等の生後の行動発生は同じ児動物を対象に検査日の間隔を均一にして観察する。

表5　検査/試験の実施時期（最小回数の測定の場合）

| 検査時期 / 検査 | 離乳前 | 思春期 (Adolescence) | 若成獣期 (Young adults) |
|---|---|---|---|
| 身体発達指標 | | | |
| 体重、臨床観察 | 週1回 | 2週に1回 | 2週に1回 |
| 脳重量 | 生後22日 | | 終了時 |
| 神経病理学的検査 | 生後22日 | | 終了時 |
| 性成熟 | — | 適宜 | — |
| 他の発達指標 | 適宜 | — | — |
| 機能／行動 | | | |
| 生後の行動発生 | 少なくとも2項目 | — | — |
| 運動活性（慣れを含む） | 1－3回（生後13, 17, 21日） | — | 1回（生後60-70日） |
| 運動・感覚機能 | — | 1回（生後23-27日） | 1回（生後60-70日） |
| 学習・記憶 | — | 1回（生後23-27日） | 1回（生後60-70日） |

運動活性は離乳前及び成熟期に測定する。無処置対照群動物の慣れが観察できるように十分な時間観察する。生後の行動発生の指標として運動活性の測定が強く推奨される。離乳前の検査は同一の動物を用い、慣れの発生が評価できるように離乳日を含めて3回以上検査する（例えば生後13、17、21日）。試験終了時近くの時期（例えば生後60～70日）の検査は同一動物または同腹児を用いる。自動記録装置を用いて運動活性を測定する。個別の動物毎に運動活性を測定する。測定装置、測定日の違いによる結果のバラツキが小さくなるように配慮する。音、ケージの形状及び大きさ、温度、明るさ、臭い、ホームケージまたは新規のケージ及び環境変化等が行動測定に影響を及ぼす。

運動及び感覚機能検査は思春期と成熟期にそれぞれ少なくとも1回行う。運動及び感覚機能として伸筋推力反応、正向反射、聴覚性驚愕反応の慣れ及び誘発電位等が例示されている。

学習記憶に関する検査は離乳後の生後23～27日及び生後60日以降の成熟期に行う。同一または別の検査を成長の2つの時期に実施する。離乳児と成熟ラットの学習記憶検査の選択は柔軟性を持って行うが、次の2つの基準、すなわち、「1）反復訓練による行動の変化として捉えうる学習であること、また、単回の訓練で学習が成立するモデルでは訓練経験をマッチさせた条件と比較すること、2）学習の獲得とともに記憶（短期記憶ないし長期記憶）の測定を組み込むこと」を満たす必要がある。学習記憶検査として、受動回避やモリス型水迷路等が例示されている。学習と記憶の検査で被験物質の影響が示されたときには、感覚、動機付けまたは運動能力の変化に基づいた変化である可能性を排除するために追加検査を考慮する。

母動物は児動物の離乳後に安楽死させる。児動物の神経病理学的検査は、生後11～22日または生後22日、さらに試験終了時に実施する。生後22日までに安楽死させた児動物について浸漬または灌流固定した脳を、試験終了時に安楽死させた児動物については灌流固定後に中枢神経系と末梢神経系を検査する。

病理組織学的検査は神経系の全ての主要な部位を反映させなければならない。特に試験終了時に計画殺された成獣について、大脳、小脳、脳幹、眼球（視神経と網膜を含む）、脊髄頚・腰膨大部、脊髄背根・腹根、坐骨神経近位部、脛骨神経近位部（膝部）及び脛骨神経腓腹分岐部）を採取する。

全ての動物について同じ位置の切片を作製することが重要である。脊髄と末梢神経は輪切りと縦断の双方を作製する。採取した組織は適切な固定液に保存し、組織学的検査の標準的な手法で標本作製を行う。採取した組織は常法に従ってパラフィン包埋を行う。末梢神経については形態計測等、より詳細な検索が要求される場合、オスミウム酸後固定を施してエポキシ樹脂包埋を行なうことが望ましい。生後22日あるいはそれ以前に安楽死させた動物の脳についてはヘマトキシリン・エオジン染色標本でよい。一方、最終計画殺動物の中枢及び末梢神経系の組織切片については、髄鞘染色（ルクソール・ファースト青/クレシール紫など）や鍍銀染色（ビルショースキーあるいはボディアン染色等）が推奨される。病理学者の専門的判断や病変の種類によっては、他の染色（抗GFAP（glial fibrillary acidic protein）抗体による免疫染色やレクチン組織化学、神経細胞壊死を特異的に検出するfluoro-jadeあるいは鍍銀染色等）も考慮する。全ての神経病理学組織的変化について重篤度を示す等級付けを行う。標本作製に当たっては、アーティファクトを避けるよう努める。

形態計測（定量）的評価は投与関連性作用の検出を助け、脳の重量や形態学的変化に対する投与関連性を解釈する上で有益である。これには、脳の特定部位の長さや面積、体積、細胞数等などの立体解析が挙げられる。

神経系の組織学的検査は熟練した病理学者が行なうべきである。細胞の変化（神経細胞の空胞化、変性、壊死等）や組織の異常（グリオーシス、白血球浸潤、海綿状変性等）とともに、神経系に対する発生障害を意識して病理組織学的評価を行う。特に、殺処分時の成長段階における正常構造と投与に関連する影響を見分けることが重要である。発生上の障害を示唆する主な例を以下に示す。嗅球、大脳あるいは小脳の大きさや形状の異常；脳の特定部位の相対的な大きさの変化（小脳外胚芽層や脳梁等）；大脳皮質における各層の相対的な厚さの変化；異所性組織、変異あるいは奇形；過度のアポトーシスあるいは壊死；移動及び分化の異常；髄鞘の厚さや染色性の変化、髄鞘形成の異常；脳室拡張、中脳水道の狭窄ならびに大脳実質の疎性化等が顕著な水頭症。

神経病理学的検査における定性的、定量的な解析については、以下のような段階を追った手順が推奨される。まず、対照群と高用量群の組織標本を比較

する。高用量群の動物に病理組織学的変化が認められない場合、それ以上の検索は不要とする。高用量群で神経病理学的変化が認められる場合、中間及び低用量群の動物について検索する。高用量群で死亡や切迫と殺があった場合は、高及び中間用量群について病理組織学的検索を行なう。低用量群において何らかの神経毒性が示唆される場合、神経病理学的検索を実施しなければならない。定性あるいは定量的検索において投与に起因する何らかの神経病理学的変化が認められる場合、全動物の検索を行い、発生頻度、程度、あるいは形態計測的変化について、用量依存性を検討する。病理組織学的検査には盲検化が推奨される。神経病理学的検査に関する更なる解説については Guidance Document No. 20 [8] を参照されたい。

### 4.4. おわりに

以上、ガイドラインに沿って概説したが、ガイドラインに記載されないその他の注意もある。例えば、自家繁殖と比べて購入した妊娠動物の胎児脳は薬物の影響を受けやすいことが報告されており [3]、妊娠動物を購入するか自家繁殖するかで異なる試験結果を得る可能性がある。交配前の雌ラットに投与したときに児動物の行動に変化を引き起こす化学物質が存在することから [14) 15)]、被験物質の毒性学的な特性に合わせた投与時期を選定する必要がある。ヒトの胎生後期はラットの哺育期に相当するため [16]、ヒトの知見からラットの試験を計画する場合やラットの結果をヒトに外挿する際には、発生段階の違いを考慮する必要がある。

神経系の発生は神経細胞の増殖、移動、シナプス形成、ミエリン形成、アポトーシス等の重要な時期が長く続き、それらの時期に応じて多岐にわたる異常が発現する可能性がある。OECD のガイドラインはこのような多岐にわたる異常を検出できるように多数の項目が記載されている。要求される検査の中で、学習と記憶や形態計測は従来の試験で求められなかった高度な知識や技術が必要となる。試験の設計や得られた結果の解釈には幅広い知識が要求される。今後は、本ガイドラインに準拠した試験の実施を通じて、その有用性を実証する段階に入ったと考える。

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# Repeated-Dose and Reproductive Toxicity of the Ultraviolet Absorber 2-(3′,5′-Di-*tert*-butyl-2′-hydroxyphenyl)-5-chlorobenzotriazole in Rats

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2-(3′,5′-Di-*tert*-butyl-2′-hydroxyphenyl)-5-chlorobenzotriazole (DBHCB) is widely used as an ultraviolet (UV) absorber. In this study, the repeated dose and reproductive toxicity of DBHCB was evaluated in rats. Crj:CD(SD)IGS rats were given DBHCB by gavage at 0, 2.5, 25, or 250 mg/kg/d. Male and female rats were dosed beginning 28 d before mating, and each female rat was mated with a male rat of the same dosage group. Males were dosed for a total of 56–57 d, and females were dosed for a total of 55–69 d up to Day 3 of lactation throughout the mating and pregnancy periods. Ten males from each group were killed on the next day of the last administration, and 10 females were killed on Days 4–6 after parturition. Five rats/sex treated at 0 and 250 mg/kg/d for 56 d were then kept without treatment for 14 d (recovery period). No deaths were found in any group. No effects of DBHCB on general condition, body weight, food consumption, or reproductive/developmental parameters were observed. Significant increases in serum albumin and an albumin/globulin ratio at 25 mg/kg/d and higher and alkaline phosphatase levels at 250 mg/kg/d were noted in males. The absolute and relative weights of the liver were significantly increased in males at 25 mg/kg/d and higher. Significantly increased serum albumin and absolute and relative liver weight were also found in males at 250 mg/kg/d after the recovery period. No changes in these parameters were observed in females of any DBHCB-treated groups. No significant changes in organ histopathology were found in males or females. These findings indicated a sex difference in the toxicity of DBHCB in rats.

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***Keywords*** Repeated-dose toxicity, Reproductive and developmental toxicity, UV absorber, Benzotriazole, Rat.

## INTRODUCTION

Benzotriazole ultraviolet (UV) absorbers, which have a phenolic group attached to the benzotriazole structure, are known to have the most excellent absorption capacity within the full spectrum of UV absorption (Tenkazai.com, 2007) and are, therefore, used in a variety of polymers. 2-(3′,5′-Di-*tert*-butyl–2′-hydroxyphenyl)-5-chlorobenzotriazole (CAS No. 3864–99–1; DBHCB), one of the benzotriazole UV absorbers, is a slightly yellowish powder that is stable under ordinary conditions and insoluble in water. The annual production and import from April 2005 to March 2006 was 532 tons in Japan (METI, 2006). This chemical provides effective light stabilization and prevents the yellowing and degradation of polymers such as polypropylene, high-density polyethylene, unsaturated polyesters, styrene-based thermoplastic elastomers, polyamides, and impact polystyrenes (Chemical Land21, 2005). The finished polymers, which contain DBHCB less than 0.5% by weight of polyethylene phthalate polymers in compliance with 21 CFR 177.1630 (FDA, 2005a), may be used in contact with foods and used under certain conditions, as described in 21 CFR 176.170 (FDA, 2000; 2005b). UV absorbers are used in food packaging to prevent polymer degradation and/or a change in the quality of the packed food due to UV light.

There is growing concern that humans have been exposed to these chemicals from environmental contamination and from the contamination of packaged food. Exposure could lead to adverse effects due to the potential toxicity of the chemicals. Important information can be gained by studying the biological effects of environmental chemicals in laboratory animals.

Only limited information on the toxicity of DBHCB is available. DBHCB was not estrogenic in a recombinant yeast assay (Miller et al., 2001) or a yeast two-hybrid assay (Kawamura et al., 2003). It has been found that the oral LD50 for DBHCB is greater than 5,000 mg/kg in rats, that DBHCB causes slight skin and eye irritation in rabbits, and that DBHCB treatment resulted in dose-dependent increases in the liver weight and signs of liver toxicity at 22–800 mg/kg/day, but not at 3.7 mg/kg/day, in rats (Everlight Chemical Industrial Corporation, 2002). We previously reported that the maternal administration of DBHCB on Days 5–19 of pregnancy caused no adverse effects in dams and fetuses at doses up to 1,000 mg/kg/day (Ema et al., 2006).

Although testing for reproductive toxicity has become an important part of the overall toxicology profile for chemicals, no report is available for the reproductive toxicity of DBHCB. The present study was, therefore, conducted by using a study design similar to the OECD Guideline 422 Combined Repeated

Dose Toxicity Study with Reproduction/Developmental Toxicity Screening Study in rats (OECD, 1996).

## MATERIALS AND METHODS

### Animals

International Genetic Standard (Crj: CD (SD) IGS) rats were used throughout this study. This strain was chosen because it is most commonly used in reproductive and developmental toxicity studies and historical control data are available. Males at 11 weeks of age and females at 10 weeks of age were purchased from Hino Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan). The rats were acclimatized to the laboratory for one week prior to the start of the experiment. Male and female rats found to be in good health were selected for use. Animals were reared on a basal diet (CE-2; CLEA Japan, Inc., Tokyo, Japan) and water *ad libitum*. Rats were maintained in an air-conditioned room at 21.5–22.1°C, with a relative humidity of 47–67%, a 12-h light/dark cycle, and ventilation with 15 air changes/h. Rats were housed individually, except during the acclimation, mating, and nursing periods. From Day 0 of pregnancy to the day of sacrifice, individual dams and litters were reared by using wooden chips as bedding (White Flake; Charles River Laboratories Japan, Inc.). This experiment was approved by the Institutional Animal Care and Use Committee of Shin Nippon Biomedical Laboratories, Ltd. (SNBL; Kagoshima, Japan) and performed in accordance with the ethics criteria contained in the bylaws of the committee of SNBL.

### Chemicals and Dosing

DBHCB was obtained from Musashino Chemical Laboratory, Ltd. (Kitaibaraki, Japan). The DBHCB (Lot no. 05004IX3) used in this study was 99.9% pure based on high-performance liquid chromatography (HPLC) analysis, and it was kept in a dark, cool place at room temperature under airtight conditions. The purity and stability of the chemical were verified by analysis before the study.

DBHCB was suspended in 5% gum arabic solution. The volume of each dose was adjusted to 10 mL/kg body weight based on the latest body weight. The control rats were given only 5% gum arabic solution. Stability of the formulations kept in a dark, cool place under airtight conditions had been confirmed for up to 14 d. During use, the formulations were maintained under these conditions for no more than seven days and were 97.3–100.1% of the target concentration.

The initial numbers of the rats were 15/sex at 0 (control) and 250 mg/kg/d, and 10/sex at 2.5 and 25 mg/kg/d. Male and female rats were dosed once-daily

beginning 28 d before mating, and each female rat was mated with a male rat of the same dosage group. Males were dosed for a total of 56–57 d, and females were dosed for a total of 55–69 days to Day 3 of lactation throughout the mating and pregnancy periods. Ten males from each group were killed after 56–57 d of administration, and ten females were killed on Days 4–6 after parturition. The remaining five rats/sex treated at 0 and 250 mg/kg/d for 56 d were kept without treatment for 14 d (recovery period). Dosage levels were determined based on the results of our dose-finding study, in which significantly increased liver weight occurred in males at 250 mg/kg/d and higher, but not in females, even at 1,000 mg/kg/day, after the administration of DBHCB for 14 d in rats.

## Observations

All rats were observed twice a day for clinical signs of toxicity during the administration period and once a day during the nonadministration period. The body weight was recorded twice a week in males, and twice a week during the premating period, on Days 0, 7, 14, and 20 of pregnancy and on Days 0, 3, and 4 of lactation in females. Food consumption was recorded twice a week for males, and twice a week during the premating period, on Days 1, 4, 7, 11, 15, 17, and 20 of pregnancy and on Days 1 and 3 of lactation for females.

Prior to scheduled terminal necropsy, blood samples for hematological and biochemical evaluation were collected from the abdominal aorta of five fasted male and female rats per group under anesthesia by an intraperitoneal injection of sodium pentobarbital. Blood samples were analyzed for the following hematological parameters by using $K_2$-EDTA as an anticoagulant: red blood cell count (RBC), white blood cell count (WBC), hematocrit value, hemoglobin concentration, platelet count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte ratio, and differential white blood cell ratio (Hematology System ADVIA 120; Bayer Diagnostics Manufacturing Ltd., Dublin, Ireland), using sodium citrate as an anticoagulant: prothrombin time (PT) and activated partial thromboplastin time (APTT) (Automated Blood Coagulation Measuring Apparatus CA-5000; Sysmex Corp., Kobe, Japan).

Serum samples obtained from centrifuged whole blood were analyzed for the following biochemistry parameters: aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine phosphokinase, total bilirubin, total protein, albumin, total cholesterol, triglyceride, glucose, blood urea nitrogen (BUN), creatinine, inorganic phosphorus, calcium, sodium, potassium, chloride (Automatic analyzer JCA-BM8; JOEL Ltd., Tokyo, Japan), total bile acid (Spectrophotometer U-3200; Hitachi Ltd., Tokyo, Japan), protein fraction (Automatic Electrophoresis Apparatus, AES-4000; Olympus Corp., Tokyo, Japan), and albumin/globulin (A/G) ratio.

At the scheduled terminal necropsy, all rats were euthanized by exsanguination under anesthesia. All rats were subjected to gross necropsy, which included an external examination of all body orifices and surfaces, and examinations of all cranial, thoracic, and abdominal organs. The brain, heart, liver, kidney, spleen, thymus, and adrenal gland in males and females, the testis, epididymis, seminal vesicle, and prostate in males, and the ovary in females were removed and weighed. Relative organ weights (mg or g/100 g of body weight) were calculated on the basis of the terminal body weight. In females, the numbers of corpora lutea and implantation sites were recorded. Samples of tissues and organs were preserved in neural phosphate-buffered 10% formaldehyde solution. The testis and epididymis were fixed in Bouin's solution. Histopathological evaluations for five rats/sex /group were performed on the tissues specified below after fixation, paraffin embedding, and sectioning and staining with hematoxylin and eosin; the brain, heart, thymus, kidney, spleen, adrenal gland, small and large intestine, lung, trachea, thyroid, submandibular and mesenteric lymph node, femur bone marrow, spinal cord, sciatic nerve, tibial nerve, urinary blabber, testis, epididymis, seminal vesicle, prostate, ovary, and uterus in the control and highest dose groups, and the liver in all groups.

Each female rat was mated overnight with a single male rat of the same dosage group until copulation occurred. During the mating period, daily vaginal smears were examined for the presence of sperm. The presence of sperm in the vaginal smear and/or a vaginal plug was considered evidence of successful mating. The day of successful mating was designated as Day 0 of pregnancy. The females were allowed to deliver spontaneously and nurse their pups until postnatal days (PNDs) 4–6. The day on which parturition was completed was designated as PND 0. Litter size and numbers of live and dead pups were recorded, and the live pups were sexed and individually weighed on PNDs 0 and 4. Dead pups were examined grossly. On PND 4, the pups were euthanized by exsanguination under anesthesia, and gross external and internal examinations were performed.

## Data Analysis

The statistical analysis of pups was carried out by using the litter as the experimental unit. The body weight, body-weight gain and food consumption, precoital interval, length of gestation, numbers of implantations and live pups per litter and pup weight, delivery index, viability index, hematological and blood biochemical parameters, and organ weight were analyzed with Bartlett's test for homogeneity of variance at the 5% level of significance. When the variance was homogeneous, Dunnett's test was performed to compare the mean value in the control group with that in each DBHCB group. When the variance was heterogeneous, a Dunnett-type test was performed to

compare the mean value in the control group with that in each DBHCB group after rank conversion. Recovery in the control and highest dose groups was analyzed in the following way. Variance ratio was analyzed by an $F$ test. If the variance ratio was equivalent, the groups were compared by a Student's $t$-test. If the variance was not equivalent, the Wilcoxon test was performed.

## RESULTS

No deaths or DBHCB-related clinical signs of toxicity were found in male or female rats of any groups. There was no significant difference in the body weight and body-weight gain between the control and DBHCB-treated groups in males and females, including during pregnancy and lactation. No significant changes in the food consumption were found, except for a significant decrease on Days 28–29 in males and an increase on Days 31–32 in females at 250 mg/kg.

The reproductive and developmental findings in rats given DBHCB are presented in Table 1. Although one pair did not copulate in the control group, all pairs copulated and all copulated females were impregnated and delivered their pups in all DBHCB-treated groups. There was no significant difference in the copulation index, fertility index, gestation index, precoital interval, or gestation length between the control and DBHCB-treated groups. No effects of DBHCB were observed on the numbers of corpora lutea or implantations, preimplantation loss, numbers of pups delivered, live pups, or stillborn or sex ratio of live pups. There was no significant difference in the viability or body weight of pups on PNDs 0 or 4 between the control and DBHCB-treated groups. External and internal examinations revealed no morphological anomalies in the pups of any group.

Table 2 shows the hematological findings in rats given DBHCB at the end of the administration period. A significantly decreased RBC at 250 mg/kg/d and shorter APTT at 25 and 250 mg/kg/d were observed in males. The number of neutrophils was significantly increased, at 250 mg/kg/d, in males. In females, the only significant change was a lowered number of eosinophils, at 25 and 250 mg/kg/d. At the end of the recovery period, significantly increased numbers of platelets and neutrophils, as well as an increased neutrophil ratio, were observed in males at 250 mg/kg/d, in addition to a decreased lymphocyte ratio.

Table 3 presents the blood biochemical findings in rats given DBHCB at the end of the administration period. In males, significantly increased levels of ALAT at 25 mg/kg/d, as well as decreased levels of creatinine at 25 mg/kg/d and higher, were observed. Additionally, males presented decreased levels of total bilirubin, and increased levels of ALP, at 250 mg/kg/d, were observed. The levels of total protein were significantly increased at 25 mg/kg/d. A significantly increased albumin percentage and A/G ratio and decreased

**Table 1:** Reproductive and developmental findings in rats given DBHCB.

| DBHCB (mg/kg/day) | 0 (control) | 2.5 | 25 | 250 |
|---|---|---|---|---|
| No. of pairs | 10 | 10 | 10 | 10 |
| Copulation index (%)[b] | 90 | 100 | 100 | 100 |
| Fertility index (%)[c] | 100 | 100 | 100 | 100 |
| No. of pregnant females | 9 | 10 | 10 | 10 |
| Precoital interval (days)[a] | 4.9 ± 4.4 | 3.4 ± 3.8 | 2.7 ± 1.3 | 2.8 ± 1.5 |
| Gestation index (%)[d] | 100 | 100 | 100 | 100 |
| Gestation length (days)[a] | 21.9 ± 0.4 | 21.9 ± 0.3 | 22.0 ± 0.4 | 22.0 ± 0.2 |
| No. of litters | 9 | 10 | 10 | 10 |
| No. of corpora lutea[a] | 16.1 ± 1.9 | 15.7 ± 1.8 | 15.3 ± 1.5 | 16.0 ± 1.9 |
| No. of implantations[a] | 15.3 ± 1.7 | 14.8 ± 1.5 | 14.1 ± 1.2 | 14.2 ± 3.2 |
| Preimplantation loss (%)[a,e] | 6.1 ± 4.3 | 6.6 ± 8.4 | 7.5 ± 7.0 | 10.9 ± 17.3 |
| Delivery index (%)[a,f] | 91.1 ± 7.2 | 93.8 ± 7.0 | 91.0 ± 13.8 | 96.5 ± 5.7 |
| No. of pups delivered[a] | 14.1 ± 2.2 | 14.0 ± 1.9 | 12.8 ± 2.0 | 14.0 ± 3.1 |
| No. of live pups[a] | 14.0 ± 2.2 | 13.9 ± 1.9 | 12.8 ± 2.0 | 13.9 ± 2.9 |
| No. of stillborn[a] | 0.1 ± 0.3 | 0.1 ± 0.3 | 0 | 0.1 ± 0.3 |
| Sex ratio of live pups (female/total)[a] | 0.53 ± 0.09 | 0.50 ± 0.15 | 0.53 ± 0.09 | 0.61 ± 0.16 |
| Viability index during lactation (%)[a,g,h] | | | | |
| Day 0 | 99.2 ± 2.4 | 99.3 ± 2.3 | 98.8 ± 3.7 | 98.8 ± 2.6 |
| Day 4 | 100 | 98.8 ± 2.6 | 97.6 ± 4.0 | 97.7 ± 3.7 |
| Male pup weight during lactation (g)[a] | | | | |
| Day 0 | 6.5 ± 0.5 | 6.5 ± 0.5 | 6.8 ± 0.3 | 6.5 ± 0.4 |
| Day 4 | 9.3 ± 1.1 | 9.4 ± 0.9 | 10.2 ± 0.7 | 9.6 ± 1.4 |
| Female pup weight during lactation (g)[a] | | | | |
| Day 0 | 6.0 ± 0.4 | 6.2 ± 0.5 | 6.3 ± 0.4 | 6.1 ± 0.4 |
| Day 4 | 8.9 ± 1.0 | 9.0 ± 0.8 | 9.7 ± 0.7 | 9.1 ± 1.5 |

[a]Values are given as the mean ± SD.
[b]Copulation index (%) = (no. of females with successful copulation/no. of females paired) × 100.
[c]Fertility index (%) = (no. of females pregnant/no. of females with successful copulation) × 100.
[d]Gestation index (%) = (no. of females that delivered live pups/no. of pregnant females) × 100.
[e]Preimplantation loss (%) = ((no. of corpora lutea - no. of implantations)/no. of corpora lutea) × 100.
[f]Delivery index (%) = (no. of pups delivered/no. of implantations) × 100.
[g]Viability index on postnatal day 0 (%) = (no. of live pups on postnatal day 0/no. of pups delivered) × 100.
[h]Viability index on postnatal day 4 (%) = (no. of live pups on postnatal day 4/no. of live pups on postnatal day 0) × 100.

α2-globulin percentage were found in males at 25 and 250 mg/kg/d, as well as a decreased percentage of β-globulin at 2.5 mg/kg/d and higher. In females, the levels of total cholesterol were significantly decreased at 2.5 and 25 mg/kg/d. No significant changes in other blood biochemical parameters were noted in males and females in the DBHCB-treated groups. At the end of the recovery period, significantly increased levels of total protein, albumin, and total cholesterol and decreased creatinine levels and α2-globulin ratio were observed at 250 mg/kg/d in males. In females, parameters remained unchanged in all DBHCB-treated groups.

**Table 2:** Hematological findings in male and female rats given DBHCB.

| DBHCB (mg/kg/day) | 0 (control) | 2.5 | 25 | 250 |
|---|---|---|---|---|
| No. of male rats | 5 | 5 | 5 | 5 |
| RBC ($10^6$/mL) | 8.18 ± 0.32[a] | 7.95 ± 0.31 | 8.07 ± 0.30 | 7.63 ± 0.36* |
| WBC ($10^3$/mL) | 9.41 ± 1.06 | 8.22 ± 2.94 | 8.10 ± 2.37 | 8.94 ± 1.13 |
| Hematocrit value (%) | 45.6 ± 1.9 | 44.3 ± 0.9 | 44.7 ± 2.2 | 42.7 ± 1.7 |
| Hemoglobin concentration (g/dL) | 15.2 ± 0.4 | 14.9 ± 0.5 | 15.1 ± 0.9 | 14.2 ± 0.7 |
| Platelet count ($10^3$/mL) | 1063 ± 110 | 1145 ± 134 | 1202 ± 119 | 1205 ± 108 |
| MCV (fL) | 55.7 ± 2.3 | 55.8 ± 1.5 | 55.4 ± 0.9 | 55.9 ± 0.7 |
| MCH (pg) | 18.7 ± 0.7 | 18.7 ± 0.8 | 18.7 ± 0.4 | 18.6 ± 0.3 |
| MCHC (g/dL) | 33.5 ± 0.7 | 33.5 ± 0.7 | 33.8 ± 0.4 | 33.3 ± 0.4 |
| Reticulocyte ratio (%) | 2.60 ± 0.34 | 2.74 ± 0.57 | 3.00 ± 0.40 | 3.02 ± 0.44 |
| PT (sec) | 8.52 ± 0.42 | 9.50 ± 0.97 | 9.20 ± 0.57 | 8.50 ± 0.58 |
| APTT (sec) | 20.1 ± 0.8 | 20.9 ± 0.7 | 18.3 ± 1.0** | 18.2 ± 0.7** |
| No. of female rats | 5 | 5 | 5 | 5 |
| RBC ($10^6$/mL) | 6.81 ± 0.49[a] | 6.90 ± 0.36 | 6.82 ± 0.14 | 6.50 ± 0.24 |
| WBC ($10^3$/mL) | 5.95 ± 0.96 | 6.19 ± 1.38 | 6.34 ± 1.46 | 5.05 ± 0.71 |
| Hematocrit value (%) | 40.2 ± 2.1 | 41.1 ± 1.7 | 39.4 ± 1.2 | 39.6 ± 2.3 |
| Hemoglobin concentration (g/dL) | 13.4 ± 0.7 | 14.0 ± 0.8 | 13.1 ± 0.4 | 13.4 ± 0.8 |
| Platelet count ($10^3$/mL) | 1468 ± 237 | 1518 ± 44 | 1496 ± 208 | 1503 ± 157 |
| MCV (fL) | 59.1 ± 2.4 | 59.6 ± 1.8 | 57.8 ± 2.1 | 60.9 ± 1.5 |
| MCH (pg) | 19.7 ± 0.8 | 20.3 ± 0.5 | 19.3 ± 0.7 | 20.5 ± 0.5 |
| MCHC (g/dL) | 33.3 ± 0.2 | 34.0 ± 0.6 | 33.4 ± 0.7 | 33.7 ± 0.4 |
| Reticulocyte ratio (%) | 6.48 ± 2.55 | 4.88 ± 1.04 | 4.48 ± 1.28 | 6.28 ± 2.55 |
| PT (sec) | 7.38 ± 0.29 | 7.28 ± 0.19 | 7.42 ± 0.27 | 6.94 ± 0.32 |
| APTT (sec) | 18.6 ± 1.2 | 19.1 ± 1.9 | 18.8 ± 0.3 | 14.7 ± 3.4 |

[a]Values are given as the mean ± SD.
*Significantly different from the control, $p < 0.05$.
**Significantly different from the control, $p < 0.01$.

The organ weights of male rats given DBHCB at the end of the administration period are presented in Table 4. The absolute and relative weights of the liver were significantly higher at 25 mg/kg/d and higher. No significant changes in the weight of the reproductive organs were found. At the end of recovery period the absolute and relative weights of the liver at 250 mg/kg/d, were still significantly increased.

Table 5 shows the organ weight of female rats given DBHCB at the end of the administration period. There were no significant changes in the absolute and relative weights of organs, including the reproductive organs. At the end of the recovery period, no significant changes in the absolute or relative weight of organs were observed at 250 mg/kg/d.

No changes related to the administration of DBHCB were found in the necropsy findings. Histopathological examinations revealed no test compound–related toxicological changes in the liver of males and females in all the DBHCB-treated groups. There were also no changes in the other organs, including the male and female reproductive organs, in the 250 mg/kg/d group.

**Table 3:** Blood biochemical findings in male and female rats given DBHCB.

| DBHCB (mg/kg/day) | 0 (control) | 2.5 | 25 | 250 |
|---|---|---|---|---|
| No. of male rats | 5 | 5 | 5 | 5 |
| ASAT (IU/L) | 116 ± 26[a] | 92 ± 18 | 136 ± 28 | 121 ± 23 |
| ALAT (IU/L) | 38.8 ± 3.7 | 39.2 ± 2.9 | 58.2 ± 25.5* | 48.8 ± 7.5 |
| ALP (IU/L) | 539 ± 57 | 476 ± 78 | 617 ± 178 | 943 ± 150** |
| Total bilirubin (mg/dL) | 0.052 ± 0.008 | 0.048 ± 0.016 | 0.046 ± 0.013 | 0.024 ± 0.009** |
| BUN (mg/dL) | 20.7 ± 1.2 | 19.7 ± 2.6 | 21.8 ± 1.9 | 21.3 ± 3.8 |
| Creatinine (mg/dL) | 0.312 ± 0.053 | 0.274 ± 0.022 | 0.226 ± 0.037** | 0.248 ± 0.022** |
| Total cholesterol (mg/dL) | 68.0 ± 6.9 | 58.4 ± 12.8 | 64.0 ± 7.3 | 61.2 ± 16.5 |
| Glucose (mg/dL) | 186 ± 14 | 173 ± 14 | 190 ± 15 | 198 ± 27 |
| Total protein (g/dL) | 5.60 ± 0.10 | 6.04 ± 0.27 | 6.26 ± 0.41** | 5.92 ± 0.034 |
| Albumin (%) | 51.5 ± 2.3 | 53.3 ± 1.8 | 58.6 ± 2.5** | 61.0 ± 1.7** |
| A/G ratio | 1.07 ± 0.10 | 1.14 ± 0.09 | 1.42 ± 0.14** | 1.57 ± 0.11** |
| α1-Globulin (%) | 20.4 ± 2.7 | 20.7 ± 2.5 | 19.1 ± 2.9 | 18.1 ± 1.2 |
| α2-Globulin (%) | 9.4 ± 0.5 | 9.0 ± 0.3 | 7.8 ± 0.2** | 7.6 ± 0.4** |
| β-Globulin (%) | 14.5 ± 0.9 | 12.9 ± 1.0** | 10.6 ± 0.6** | 9.0 ± 0.3** |
| γ-Globulin (%) | 4.2 ± 1.0 | 4.2 ± 0.3 | 4.0 ± 0.8 | 4.2 ± 0.8 |
| No. of female rats | 5 | 5 | 5 | 5 |
| ASAT (IU/L) | 130 ± 11[a] | 113 ± 37 | 106 ± 15 | 104 ± 23 |
| ALAT (IU/L) | 59.0 ± 9.1 | 42.8 ± 7.8 | 49.4 ± 9.9 | 60.2 ± 15.3 |
| ALP (IU/L) | 215 ± 29 | 185 ± 71 | 184 ± 56 | 194 ± 59 |
| Total bilirubin (mg/dL) | 0.058 ± 0.016 | 0.074 ± 0.030 | 0.044 ± 0.011 | 0.056 ± 0.013 |
| BUN (mg/dL) | 26.1 ± 8.2 | 17.3 ± 5.3 | 19.8 ± 4.1 | 18.9 ± 5.0 |
| Creatinine (mg/dL) | 0.308 ± 0.044 | 0.290 ± 0.040 | 0.330 ± 0.029 | 0.282 ± 0.028 |
| Total cholesterol (mg/dL) | 79.6 ± 16.8 | 58.4 ± 3.2* | 57.6 ± 13.3* | 64.2 ± 12.9 |
| Glucose (mg/dL) | 109 ± 16 | 109 ± 13 | 120 ± 7 | 115 ± 24 |
| Total protein (g/dL) | 5.74 ± 0.31 | 5.60 ± 0.27 | 5.54 ± 0.36 | 5.50 ± 0.22 |
| Albumin (%) | 55.0 ± 1.8 | 54.2 ± 2.1 | 55.5 ± 0.8 | 55.4 ± 1.8 |
| A/G ratio | 1.23 ± 0.09 | 1.19 ± 0.10 | 1.25 ± 0.04 | 1.25 ± 0.09 |
| α1-Globulin (%) | 17.8 ± 2.1 | 19.2 ± 1.4 | 17.8 ± 2.2 | 17.6 ± 1.3 |
| α2-Globulin (%) | 8.8 ± 1.2 | 8.8 ± 0.9 | 7.9 ± 0.8 | 8.3 ± 0.3 |
| β-Globulin (%) | 13.5 ± 0.9 | 13.3 ± 0.9 | 13.7 ± 0.8 | 13.4 ± 1.0 |
| γ-Globulin (%) | 4.9 ± 1.2 | 4.4 ± 0.4 | 5.1 ± 0.5 | 5.3 ± 0.4 |

[a]Values are given as the mean ± SD.
*Significantly different from the control, $p < 0.05$.
**Significantly different from the control, $p < 0.01$.

## DISCUSSION

The present study was conducted to determine the repeated-dose and reproductive toxicity of DBHCB. The data show that the repeated oral dosing of DBHCB caused changes in the liver in males, but not in females, and no changes in the reproductive function of male and female rats.

In the present study, there were no changes in the reproductive parameters regarding copulation, fertility, parturition, and nursing of their pups in rats given DBHCB beginning 28 d before mating, during pregnancy, and shortly after parturition. No changes in weight or histopathology were found in male and female reproductive organs. Moreover, the prenatal and postnatal

**Table 4:** Organ weights of male rats given DBHCB.

| DBHCB (mg/kg/day) | 0 (control) | 2.5 | 25 | 250 |
|---|---|---|---|---|
| No. of male rats | 5 | 5 | 5 | 5 |
| Body weight (g) | 451 ± 35[a] | 463 ± 26 | 454 ± 37 | 437 ± 11 |
| Brain (g) | 2.06 ± 0.07[b] | 2.09 ± 0.06 | 2.06 ± 0.11 | 2.00 ± 0.09 |
| | 0.461 ± 0.034[c] | 0.453 ± 0.034 | 0.457 ± 0.061 | 0.459 ± 0.030 |
| Heart (g) | 1.41 ± 0.07[b] | 1.52 ± 0.11 | 1.44 ± 0.16 | 1.42 ± 0.11 |
| | 0.314 ± 0.011[c] | 0.329 ± 0.018 | 0.312 ± 0.018 | 0.325 ± 0.024 |
| Thymus (mg) | 391 ± 90[b] | 401 ± 104 | 412 ± 174 | 396 ± 88 |
| | 87.4 ± 22.7[c] | 86.2 ± 18.6 | 89.4 ± 31.5 | 90.8 ± 20.5 |
| Liver (g) | 14.81 ± 1.43[b] | 16.46 ± 1.70 | 20.11 ± 3.76* | 24.11 ± 2.60** |
| | 3.28 ± 0.13[c] | 3.54 ± 0.20 | 4.41 ± 0.55* | 5.52 ± 0.66** |
| Kidney (g) | 3.17 ± 0.25[b] | 3.49 ± 0.31 | 3.50 ± 0.40 | 3.34 ± 0.18 |
| | 0.706 ± 0.082[c] | 0.753 ± 0.051 | 0.769 ± 0.045 | 0.763 ± 0.045 |
| Spleen (mg) | 853 ± 82[b] | 957 ± 205 | 908 ± 218 | 790 ± 62 |
| | 190 ± 19[c] | 206 ± 38 | 199 ± 35 | 181 ± 17 |
| Adrenal (mg) | 61.2 ± 9.5[b] | 62.1 ± 9.2 | 61.5 ± 6.9 | 50.8 ± 3.2 |
| | 13.6 ± 2.3[c] | 13.4 ± 2.1 | 13.6 ± 2.2 | 11.6 ± 0.6 |
| Testis (g) | 3.23 ± 0.14[b] | 3.39 ± 0.17 | 3.01 ± 0. 28 | 3.00 ± 0.25 |
| | 0.720 ± 0.062[c] | 0.732 ± 0.051 | 0.666 ± 0.073 | 0.686 ± 0.058 |
| Epididymis (g) | 1.26 ± 0.07[b] | 1.27 ± 0.06 | 1.23± 0.14 | 1.24 ± 0.13 |
| | 0.281 ± 0.027[c] | 0.274 ± 0.010 | 0.271 ± 0.018 | 0.284 ± 0.028 |
| Seminal vesicle (g) | 1.71 ± 0.18[b] | 1.69 ± 0.14 | 1.70 ± 0.21 | 1.60 ± 0.12 |
| | 0.383 ± 0.060[c] | 0.365 ± 0.040 | 0.376 ± 0.040 | 0.366 ± 0.027 |
| Prostate (g) | 1.37 ± 0.099[b] | 1.25 ± 0.10 | 1.42 ± 0.34 | 1.39 ± 0.19 |
| | 0.305 ± 0.035[c] | 0.270 ± 0.025 | 0.309 ± 0.057 | 0.319 ± 0.038 |

[a]Values are given as the mean ± SD.
[b]Absolute organ weight.
[c]Relative organ weight = organ weight (g or mg)/100 g body weight.
*Significantly different from the control, $p < 0.05$.
**Significantly different from the control, $p < 0.01$.

developmental parameters regarding embryonic/fetal/neonatal survival and growth and morphological development of offspring were not affected by the administration of DBHCB. These results are consistent with the results of our previous study, in which no maternal or prenatal developmental toxicity was noted in rats given DBHCB by gavage on Days 5–19 of pregnancy at 1,000 mg/kg/d (Ema et al., 2006). These findings indicate that DBHCB has no potential for reproductive or developmental toxicity in rats.

On the hematological examination, changes in some parameters were noted in both male and female rats at higher doses. However, these changes are not considered to indicate toxicological significance because they were relatively small and were dose independent. The lowered RBC, for example, in males at 250 mg/kg/d is unlikely to represent anemia because the degree of decrease is slight and other anemic parameters, such as hematocrit, hemoglobin, MCV, MCH, MCHC, and reticulocyte count, were not affected by the administration of DBHCB. Anemia is defined clinically as the condition characterized by a hemoglobin concentration below the lower reference limit (Hall, 2007). Regarding renal function, it has been described that serum

**Table 5:** Organ weights of female rats given DBHCB.

| DBHCB (mg/kg/day) | 0 (control) | 2.5 | 25 | 250 |
|---|---|---|---|---|
| No. of female rats | 5 | 5 | 5 | 5 |
| Body weight (g) | 282 ± 33[a] | 290 ± 14 | 276 ± 15 | 283 ± 21 |
| Brain (g) | 1.96 ± 0.04[b] | 1.96 ± 0.06 | 1.97 ± 0.09 | 1.94 ± 0.06 |
| | 0.706 ± 0.100[c] | 0.677 ± 0.044 | 0.716 ± 0.068 | 0.688 ± 0.058 |
| Heart (g) | 1.06 ± 0.14[b] | 1.00 ± 0.04 | 0.98 ± 0.08 | 1.01 ± 0.10 |
| | 0.376 ± 0.019[c] | 0.346 ± 0.012 | 0.357 ± 0.022 | 0.358 ± 0.035 |
| Thymus (mg) | 219 ± 40[b] | 272 ± 60 | 247 ± 87 | 253 ± 64 |
| | 77.6 ± 7.8[c] | 93.6 ± 18.6 | 90.2 ± 32.9 | 90.2 ± 26.5 |
| Liver (g) | 9.89 ± 1.64[b] | 8.99 ± 0.67 | 9.16 ± 0.69 | 9.69 ± 0.54 |
| | 3.51 ± 0.37[c] | 3.10 ± 0.19* | 3.32 ± 0.10 | 3.43 ± 0.20 |
| Kidney (g) | 2.16 ± 0.28[b] | 2.07 ± 0.14 | 1.98 ± 0.21 | 2.03 ± 0.03 |
| | 0.770 ± 0.067[c] | 0.713 ± 0.033 | 0.715 ± 0.057 | 0.721 ± 0.049 |
| Spleen (mg) | 716 ± 178[b] | 713 ± 125 | 666 ± 172 | 749 ± 62 |
| | 252 ± 44[c] | 246 ± 47 | 240 ± 52 | 265 ± 16 |
| Adrenal (mg) | 95.7 ± 15.2[b] | 85.0 ± 10.3 | 85.3 ± 10.3 | 89.5 ± 4.0 |
| | 34.1 ± 5.23[c] | 29.3 ± 3.6 | 30.9 ± 3.3 | 31.7 ± 1.6 |
| Ovary (mg) | 95.9 ± 10.4[b] | 96.4 ± 6.2 | 95.6 ± 11.6 | 104.9 ± 18.8 |
| | 34.5 ± 5.9[c] | 33.2 ± 1.9 | 34.7 ± 4.2 | 36.9 ± 4.5 |

[a]Values are given as the mean ± SD.
[b]Absolute organ weight.
[c]Relative organ weight = organ weight (g or mg)/100 g body weight.
*Significantly different from the control, $p < 0.05$.

creatinine levels parallel changes in BUN caused by alterations in renal blood flow, renal function, or urinary outflow (Hall, 2007). The changes in creatinine levels in male rats at 25 mg/kg/d and higher are not thought to have toxicological significance because there were no changes in BUN or histopathological alterations of the kidney in the DBHCB-treated groups. In male rats, changes in some blood biochemical parameters suggestive of liver toxicity were observed at higher doses. The increased levels of total protein and albumin suggest an acceleration of protein synthesis in the liver, and these phenomena are supported by the increased weight of the liver at higher doses. These changes were noted only in males, indicating a sex difference in the toxicity of DBHCB.

The no observed adverse effect level (NOAEL) for repeated-dose toxicity of DBHCB is considered to be 2.5 mg/kg/d in male rats, based on the increased levels of albumin and weight of the liver, and 250 mg/kg/d, the highest dose used in the present study, in female rats. Our findings indicate that male rats have more than a 100-fold greater susceptibility to DBHCB toxicity than female rats. Previously, we showed sex differences in toxicity in the 28-d and 52-week repeated-dose toxicity studies of a structurally similar compound, 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (HDBB), which is also used as a UV absorber (Hirata-Koizumi et al., 2007, 2008a). In the 28-d repeated-dose toxicity study, using rats given HDBB by gavage at 0, 0.5, 2.5,

12.5, or 62.5 mg/kg/d, adverse effects on the liver and heart were noted at all doses in males and at 12.5 mg/kg and higher in females (Hirata-Koizumi et al., 2007). In the 52-week repeated-dose toxicity study with rats given HDBB by gavage at 0, 0.1, 0.5, or 2.5 mg/kg/d in males and 0, 0.5, 2.5, or 12.5 mg/kg/d in females, toxic effects were observed in the liver at 0.5 mg/kg/d and higher in males and 12.5 mg/kg/d in females (Hirata-Koizumi et al., 2008a).

It has been recognized that there are sex differences in the toxicity of chemical compounds in rats. A recent subchronic toxicity study showed that fluoranthene, a polycyclic aromatic hydrocarbon, had greater effects on males than females, especially in the kidney, in F344 rats (Knuckles et al., 2004). On the other hand, female rats exhibited a higher susceptibility to hypothermic effects and inhibition of hypothalamic cholinesterase by the carbamate cholinesterase inhibitor, rivastigmine (Wang et al., 2001). These findings suggest that sexual hormones may play an important role in sex differences in toxicity. It has already been shown that orchidectomy resulted in the complete ablation of the above-mentioned sex differences in hypothalamic cholinesterase inhibition induced by rivastigmine (Wang et al., 2001). Testosterone is likely to interfere with the effects of rivastigmine, because testosterone decreases cholinesterase inhibition in gonadectomized males and females. More recently, we showed that castration markedly reduced sex differences in the toxicity of HDBB in male and female rats (Hirata-Koizumi et al., 2008b). We also reported no sex differences in susceptibility to the toxic effects of HDBB in preweaning rats (Hirata-Koizumi et al., 2008c). It is important to investigate the role of sex steroids in the mediation of sex differences in susceptibility to DBHCB toxicity and to determine the toxic effects of DBHCB in preweaning rats. A repeated-dose toxicity study of DBHCB is currently in progress, using castrated and preweaning male and female rats.

To date, there has been no available data for human exposure to this chemical. Actual human exposure to DBHCB may be very low because it was not detected in polyethylenterephthalate bottles in Brazil (Monteiro et al., 1998) or polyethylene products in Japan (Kawamura et al., 1997). Consideration of these findings and the results of the present study together suggest that the human risk of adverse effects from DBHCB exposure is very low.

## CONCLUSIONS

In conclusion, the administration of DBHCB during premating, mating, and pregnancy, as well as shortly after parturition, caused no changes in the reproductive function of male and female rats. DBHCB produced increases in the liver weight, albumin levels, and A/G ratio at 25 mg/kg/d and higher, as well as ALP levels at 250 mg/kg/d in males, but no change in females. These findings indicate a sex difference in the toxicity of DBHCB in rats.

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# Two-generation reproductive toxicity study of the flame retardant hexabromocyclododecane in rats

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**Abstract**

Male and female rats were fed a diet containing flame retardant hexabromocyclododecane (HBCD) at 0, 150, 1500 or 15,000 ppm throughout the study beginning at the onset of a 10-week pre-mating period and continuing through the mating, gestation and lactation periods for two generations. The mean daily intakes of HBCD during the whole period of administration were 10.2, 101 and 1008 mg/kg bw in F0 males, 14.0, 141 and 1363 mg/kg bw in F0 females, 11.4, 115 and 1142 mg/kg bw in F1 males, and 14.3, 138 and 1363 mg/kg bw in F1 females for 150, 1500 and 15,000 ppm, respectively. The incidence of rats with decreased thyroid follicles size was increased in F0 and F1 males and females at 1500 ppm and higher. Serum TSH levels were increased in F0 and F1 females at 1500 ppm and higher, and serum T4 levels were decreased in F0 males and females at 15,000 ppm. The number of the primordial follicles in the ovary of F1 females was reduced at 1500 ppm and higher. There were increases in the absolute and relative weights of the liver in male adults and male and female weanlings at 1500 ppm and higher, and in female adults at 15,000 ppm, and of the thyroid in male and female adults at 15,000 ppm. Decreased body weight and body weight gain associated with reduced food consumption were found in F1 males and females at 15,000 ppm. Decreases were found in the viability index of F2 pups and the body weight of male F1 and F2 pups and female F2 pups at 15,000 ppm. In F2 pups, there were low incidences of the completion of eye opening in males at 15,000 ppm and in females at 1500 ppm and higher, and of completed mid-air righting in females at 15,000 ppm. The data indicate that the NOAEL of HBCD in this study was 150 ppm (10.2 mg/kg bw/day). The estimated human intake of HBCD is well below the NOAEL in the present study.



*Keywords:* Hexabromocyclododecane; Brominated flame retardant; Two-generation reproductive toxicity; Developmental toxicity; Rat

## 1. Introduction

Although about 80 different brominated organic flame retardants are registered, tetrabromobisphenol A, the polybrominated diphenyl ethers and hexabromocyclododecane (HBCD) account for most of the total volume [1]. HBCD is a nonaromatic, brominated cyclic alkane used as an additive flame retardant. Total market demand for HBCD in 2001 was estimated as 2800 tons in America, 9500 tons in Europe, 3900 tons in Asia and 500 tons in the rest of the world [2]. The commercial product is a mixture of three stereoisomers, alpha, beta and gamma, which are typically present at approximately 6, 8 and 80%, respectively [3]. Its primary application is in extruded (XPS) and expanded (EPS) polystyrene foam that is used as thermal insulation in the building industry. HBCD is the only suitable flame retardant for these applications. A secondary, although important, application of HBCD is as a flame retardant for upholstery textiles [3,4]. The partition coefficient (Log Kow) value of 5.6 suggests that this chemical is suspected to have high bioaccumulation potential [4]. HBCD has been used for about 20 years, and is detected in practically all environmental media [5]. HBCD was identified in sediment from several places along the River Viskan in Sweden [6] and the River Cinca in Spain [7]. HBCD was detected in fishes, pike (*Esox lucius*) [6] and barbel (*Barbus graellsi*) [7], indicating that it is bioavailable and bioaccumulates. The bioconcentration factor of this compound is reported to be 18,100 in fathead minnow (*Pimephales promelas*) [8]. HBCD was also detected from common whelk (*Buccinium undatum*), sea star (*Asterias rubens*), hermit crab (*Pagurus bernhardus*), gadoid fish species whiting (*Merlangius merlangus*), cod (*Gadus morhua*),

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harbor seal (*Phoca vitulina*) and harbor porpoise (*Phocoena phocoena*) from the North Sea [9]. These findings show evidence of HBCD bioaccumulation at the trophic level and biomagnification in the ascending aquatic food chain [9]. As a result of widespread use and the physical and chemical properties, HBCD is now considered to be a ubiquitous contaminant in the environment and humans [5,10]. It could be hypothesized that food intake is the largest single source of human exposure to HBCD [11].

HBCD was detected at ranging from 0.3 to 20 μg/g lipid in 49 samples of the 85 human breast milk samples collected from Norway between 1993 and 2001 [12]. The concentration of HBCD in the Stockholm human milk showed a fluctuating increase over time, and from 1980 the concentration increased from 0.13 pmol/g lipid to 0.60 pmol/g lipid in 2004 [13]. The HBCD concentration of human milks collected in 2002 to 2003 from North America was ranging from 0.3 to 10 μg/g lipid [14]. The presence of such a chemical compound in biological systems has aroused great concern about its toxicological potential. The biological effects produced by chemicals should be studied in laboratory animals to investigate their possible influences on human health, and the results of animal tests of chemical toxicity are relevant to humans [15]. The toxic effects of HBCD are briefly summarized by NRC [4], American Chemical Council [3], de Wit [16], Darnerud [11], Birnbaum and Staskal [17]. However, information on the effects of HBCD is insufficient to assess the overall toxicity of this compound. Following oral administration to male rats, HBCD was rapidly absorbed from the gastrointestinal tract, distributed primarily to the body fat, and eliminated rapidly, primarily in the feces [4]. In a 28-day repeated dose toxicity study, no toxic effects were noted in male and female SD rats at any dose of HBCD given by gavage at up to 1000 mg/kg bw/day [18]. In a 90-day repeated dose toxicity study in SD rats given HBCD at 0, 100, 300, or 1000 mg/kg bw/day by gavage, increased weights of the liver and prostate, and γ-glutamyltransferase, and decreased weight of the thyroid/parathyroid were found [19]. The author of this study concluded that these changes were probably of limited, if any, toxicological significance, because they were reversible, and not associated with specific target organ damage or diminished function. The dose-related effects of HBCD on the thyroid hormone axis were observed in a recent 28-day repeated dose study (OECD407) enhanced for endocrine and immune parameters using Wistar rats dosed by gavage at 0–200 mg/kg bw/day [20]. After a single dose of HBCD by gavage at 0.9 or 13.5 mg/kg bw by gavage on postnatal day (PND) 10, spontaneous activity and learning and memory in the water maze were altered when tested at the age of 3 months in NMRI mice [21]. As for the developmental toxicity of HBCD, two studies are available. There was no maternal or developmental toxicity in SD rats given HBCD by gavage on days 6–19 of pregnancy at any doses up to 1000 mg/kg bw/day [22]. No maternal or developmental toxicity was noted in Wistar rats given HBCD in diet at up to 1% (equivalent to 600 mg/kg bw/day) on days 0–20 of pregnancy [23]. No reproductive difficulties in dams or postnatal development in offspring were found even at the highest dose.

Although the testing for reproductive toxicity in an animal model is an important part of the overall toxicology, no information is available for the reproductive toxicity of HBCD at the present time; therefore, a two-generation reproductive toxicity study was conducted.

## 2. Materials and methods

This study was performed in 2005–2006 at the Safety Research Institute for Chemical Compounds Co., Ltd. (Sapporo, Japan) in compliance with the OECD guideline 416 Two-generation Reproduction Toxicity Study [24]. This study was conducted in accordance with the principles for Good Laboratory Practice [25], "Law for the Humane Treatment and Management of Animals" [Law No. 105, October 1, 1973, revised December 22, 1999, Revised Law No. 221; revised June 22, 2005, Revised Law No. 68], "Standards Relating to the Care, Management and Refinement of Laboratory Animals" [Notification No. 88 of the Ministry of the Environment, Japan, April 28, 2006] and "Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in the Testing Facility under the Jurisdiction of the Ministry of Health, Labour and Welfare" [Notification No. 0601005 of the Health Sciences Division, Ministry of Health, Labour and Welfare, Japan, June 1, 2006].

### 2.1. Chemical and dosing

Hexabromocyclododecane (HBCD; 1,2,5,6,9,10-hexabromocyclododecane; CAS No. 3194-55-6) was obtained from Wildlife International, Ltd. (Easton, MD). The test substance was a composite of HBCD commercial products from Albemarle Corporation (Baton Rouge, LA), Great Lakes Chemical Corporation (West Lafayette, IN) and Ameribrom Inc. (New York, NY), and Wildlife International, Ltd. prepared the composite. The preparation of HBCD was a mixture of three enantiomers. HBCD-α, HBCD-β and HBCD-γ, and their respective proportions in the used batch were 8.5, 7.9 and 83.7%. The HBCD (test substance number # 7086) used in this study was 99.7% pure, and was kept in a sealed container under cool (2–7 °C) and dark conditions. The purity and stability of the chemical were verified by analysis using liquid chromatography before and after the study.

Rats were given dietary HBCD at a concentration of 0 (control), 150, 1500 or 15,000 ppm. The dosage levels were determined based on the results of a previous 90-day oral repeated dose toxicity study [19] in male and female Crl:CD(SD)IGS BR rats given HBCD at 0, 100, 300 or 1000 mg/kg bw/day for 90 days. The author concluded that all test article-related changes, even at 1000 mg/kg bw/day, were reversible, not associated with specific target organ damage or diminished function (data not shown).

Dosed diet preparations were formulated by mixing HBCD into an appropriate amount of a powdered basal diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) for each dietary concentration. The control rats were fed a basal diet only. Analysis showed that the HBCD was homogeneous in the diet and stable for at least 21 days at room temperature, and was administered at the desired feed concentrations throughout the study.

### 2.2. Animals and housing conditions

Crl:CD(SD) rats were used throughout this study. Rats of this strain were chosen because they are the most commonly used in reproductive and developmental toxicity studies, and historical control data are available. Male and female rats at 4 weeks of age were purchased from Tsukuba Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan). The males and females were acclimated to the laboratory for 7 days prior to the start of the experiment. Male and female rats found to be in good health were selected for use. One hundred and ninety-two rats were randomly assigned 24/sex/group as F0 animals, and all animals were assigned a unique number and ear tattooed prior to the start of the experiment. Animals were housed individually in suspended aluminum/stainless steel cages, except during the acclimation, mating and nursing periods. From day 17 of pregnancy to the day of weaning, individual dams and litters were reared using wood chips as bedding (White Flake, Charles River Laboratories Japan, Inc.).