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Research article

Evaluating toxic endpoints of exposure to a commercial PCB mixture: an *in vivo* laboratory study

Herve Aloysius [1,2], Daniel Desaulniers [3] and Stelvio M. Bandiera [1,*]

[1] Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3

[2] Merck & Co., Inc., Whitehouse Station, NJ 08889, USA

[3] Environmental Health Science and Research Bureau, Health Canada, Ottawa, Ontario, Canada K1A 0K9

* **Correspondence:** Email: bandiera@mail.ubc.ca; Tel: (604) 822-3815.

Abstract: Polychlorinated biphenyls (PCBs) are ubiquitous environmental contaminants that produce a wide range of toxic effects. To determine sensitive endpoints in various organ systems, the effects of Aroclor 1260 on immune, endocrine, and hepatic systems were evaluated in a dose-response study. Nine-week old male rats were treated with Aroclor 1260 by oral gavage at dosages ranging from 0.025 to 156 mg/kg/day for 10 consecutive days and killed two days after the last treatment. Eight days prior to sacrifice, rats were injected i.v. with sheep red blood cells (SRBC) for determination of humoral immunity. No observable adverse effect level (NOAEL) and lowest observable adverse effect level (LOAEL) were determined for liver, thymus and genital organ weights, body weight, serum luteinizing hormone (LH), testosterone, thyroxine and thyroid-stimulating hormone (TSH) concentrations, hepatic microsomal testosterone hydroxylase activities, and hepatic microsomal cytochrome P450 (CYP) 1A1, CYP1A2, CYP2B1 and CYP2B2 protein levels. Treatment with Aroclor 1260, at all dosages, had no effect on testis, seminal vesicle or ventral prostate weights, on thymus weight or on serum LH or testosterone levels. Among the endpoints altered by Aroclor 1260, the most sensitive, with a LOAEL of 1.25 mg/kg/day, were increased testosterone 16β-hydroxylase activity and androstenedione formation. The LOAEL for increased liver weight, testosterone 16α-hydroxylase activity and CYP2B1 protein content was 3.13 mg/kg/day, while the LOAEL for decreased serum thyroxine levels and anti-SRBC IgM titer was 6.25 mg/kg/day. Less sensitive responses, as reflected by larger LOAEL values, included CYP1A enzyme induction and decreased body weight. In summary, comparison of NOAEL and LOAEL values indicated that hepatic CYP2B-mediated activities were a more sensitive response to Aroclor 1260 exposure in male rats than immune or endocrine endpoints.

Keywords: polychlorinated biphenyls; Aroclor 1260; immune suppression; thyroid hormones; rat liver; CYP induction; testosterone hydroxylase activities

1. Introduction

Polychlorinated biphenyls (PCBs) were among the first chemicals to be labeled as persistent organic pollutants by the World Health Organization. PCBs are no longer produced commercially but these compounds are still found in older closed systems such as electrical transformers and continue to be released into the environment from diverse disposal sites worldwide [1-5]. Humans and wildlife continue to be exposed to PCBs present in food chains, water and ambient air [6,7,8], even as PCB levels in the environment are gradually declining. As a result, PCBs are routinely detected in human adipose tissue, blood, and breast milk and in other biota, and often at higher levels than those of other organochlorine pollutants [9,10]. Consequently, PCBs remain a health concern at the local and international levels.

Adverse health effects associated with PCB exposure in humans include alterations of the endocrine system, immunosuppression, neurotoxicity and developmental toxicity [11-14]. In addition, the International Agency for Research on Cancer lists PCBs as probable human carcinogens [15] and recent data collected from epidemiological and public health studies suggest that exposure to PCBs can produce impairment of the auditory system [16,17]. Among endocrine systems affected by PCBs, the best characterized is the thyroid hormone system. Marked decreases in serum thyroxine (T4) concentrations have been reported in laboratory animals following chronic or short-term exposure to commercial PCB mixtures or individual PCB congeners [18-20]. Changes in circulating T4 levels have also been observed in wildlife exposed either experimentally or environmentally to PCBs [21]. Sustained alteration of circulating thyroid hormone levels has serious health implications because thyroid hormones are essential in brain development and can impact the hypothalamic-pituitary-gonadal axis, resulting in neuro-behavioral and reproductive effects [22,23,24]. Studies in rodents have shown that PCBs can modify sex hormone and luteinizing hormone (LH) levels [19,25-28]. Immune system effects associated with PCB exposure include reduced thymus and spleen weight, alterations in thymus cellularity, and diminished humoral and cell-mediated immune responses [29-33]. The higher chlorinated PCB mixtures such as Aroclor 1254 and 1260 and the coplanar congeners appear to be more immunotoxic than the lower chlorinated mixtures and non-coplanar congeners. Long-term exposure of primates to Aroclor 1254 at dosages of 5–80 μg/kg/day for 23 months resulted in a dose-dependent decline of lymphocyte proliferation and antibody response to sheep red blood cells (SRBC), effects associated with altered T-cell and macrophage function [32,34].

PCBs are found in environmental samples as mixtures of structurally diverse congeners that differ with respect to their biological and toxic effects and mechanism of action. Coplanar, non-*ortho*-substituted congeners elicit a set of adverse effects associated with high affinity binding to the aryl hydrocarbon (Ah) receptor resulting in cytochrome P450 1A (CYP1A) induction, thymic atrophy, a wasting syndrome, and skin lesions [11,35]. Non-coplanar di-*ortho*-substituted congeners bind weakly (or not at all) to the Ah receptor, induce CYP2B enzymes, and have been linked to neurotoxicity and immune toxicity [11,35].

Risk assessment analysis of complex PCB mixtures requires data that is most usefully obtained from dose-response studies involving multiple endpoints. Information about the relative sensitivity of various endpoints such as endocrine disruption or immune suppression would be helpful for

predicting the type of toxicity that might be expected at different exposure levels. Comparative information about the relative sensitivity of different responses can be obtained from published studies, but these studies often involve single PCB congeners and differ with respect to the species tested, dosages used, and duration and route of exposure. No observable adverse effect level (NOAEL) and lowest observable adverse effect level (LOAEL), which are routinely used in the risk assessment process to determine exposure levels expected to pose minimal risk to humans, are often based on carefully conducted animal studies.

The present study was undertaken to determine the relative sensitivity of functional responses to PCB exposure over a wide range of dosages within the same experimental design. Adult male rats were treated for ten days with Aroclor 1260, a technical PCB mixture that contains a relatively high concentration of non-coplanar di-*ortho*-substituted PCB congeners such as those found in environmental samples. Responses including anti-SRBC IgM titer, thyroid hormone levels, hepatic microsomal CYP1A and CYP2B enzyme content and testosterone hydroxylase activities were measured and corresponding NOAEL and LOAEL were determined.

2. Materials and methods

2.1. Reagents

Aroclor 1260 (Monsanto Chemical Co., St. Louis, MO) was a gift from Dr. S.H. Safe (Texas A&M University, College Station, TX). The Aroclor 1260 sample was from the same batch as that used and analyzed previously [36]. The congener composition of the Aroclor 1260 sample was very similar to the composition of the Aroclor 1260 reported by Frame et al. [37] and Newman et al. [38]. Corn oil was purchased from Sigma Chemical Co. (St. Louis, MO). Mouse anti-rat CYP1A monoclonal IgG, CD 5.2 Mab, that recognizes both CYP1A1 and CYP1A2 was provided by Dr. Paul E. Thomas (Rutgers-The State University of New Jersey, Piscataway, NJ). Testosterone and testosterone metabolites were purchased from Steraloids Inc. (Wilton, NH). All other reagents were obtained from sources indicated elsewhere [39].

2.2. Animals

Male Long-Evans rats weighing 250 to 300 g (7–8 weeks old) were purchased from Charles River Canada, Inc. (St-Constant, Quebec, Canada) and were allowed to acclimatize in our animal facility for 7 days prior to initiation of treatment. Rats were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care under an approved protocol of The University of British Columbia. Rats were housed two per cage in polycarbonate cages on corn-cob bedding and were given Certified Rodent Diet (5002, PMI® Feeds, Inc., Richmond, IN) and water *ad libitum*. The animal room was maintained on 12-h light and 12-h dark cycles at a controlled temperature.

2.3. Preparation of SRBC membrane antigens

Diluted sheep blood (1:1 in Alsever's solution), purchased from Colorado Serum Co. (Denver, CO), was spun at 1000 × g for 15 min and the plasma removed carefully. Cells were washed with 0.9% saline solution, spun at 1000 × g for 10 min, and re-suspended in 0.05 M Tris-HCl buffer, containing 0.1 mM EDTA, pH 7.6, as described by Temple et al. [40]. Cells from 25 mL of sheep blood were

suspended in 40 mL of Tris-HCl buffer. This procedure was repeated four times to remove all visible traces of hemoglobin. The pellet was then washed with 0.9% saline solution, spun at 1000 × g for 10 min, and re-suspended in 2.5 mL of 0.1% sodium dodecyl sulfate. Cells were counted using a hemocytometer and dilutions were made to obtain 2×10^8 SRBC per 0.5 mL. The protein concentration of the SRBC membrane preparation was measured using the bicinchoninic acid assay kit supplied by Pierce (Rockford, IL).

2.4. Animal treatment and collection of blood and tissues

Rats were divided into groups containing six animals each and were treated with Aroclor 1260 in corn oil (2.5 mL/kg/day) by oral gavage at dosages of 0.025, 0.13, 0.625, 1.25, 3.13, 6.25, 15.6, 31.3, 78.1, and 156 mg/kg/day. A control group was treated with the corn oil vehicle at a dosage of 2.5 mL/kg/day. Animals were treated once daily for ten days and killed two days after the last treatment. Body weights were measured before each treatment. On the fourth day of treatment, rats were injected with SRBC (2×10^8 cells/rat) by i.v. tail injection. Blood samples were drawn on day 10 for anti-SRBC IgM determination. Trunk blood for hormone assays was collected immediately after decapitation. Liver, thymus, testes, seminal vesicle, and ventral prostate were carefully dissected from each rat at the time of death, blotted dry, and weighed. Hepatic microsomes were prepared by differential centrifugation as described [41], suspended in 0.25 M sucrose, and stored at −75 °C.

The duration of PCB treatment was chosen to match previous work performed in our laboratory [36]. A broad range of dosages was chosen to obtain complete dose-response curves for the endpoints investigated. Dosages were selected at five-fold increments starting at 25 μg/kg/day. Additional dosages were included to help define midpoints of the dose-response relationships for specific responses where the greatest changes were expected. The highest dosage selected for the present study was 156.25 mg/kg/day, which is equal to a cumulative dose of 1.56 g/kg and is within the range of the LD_{50} values of 1.26 and 2.0 g/kg that have been reported for Aroclor 1260 [11].

2.5. Determination of total CYP and protein concentration

The total CYP concentration of hepatic microsome samples was measured from the sodium dithionite-reduced carbon monoxide difference spectrum as described by Omura and Sato [42]. The protein concentration of hepatic microsomal samples was measured by the method of Lowry et al. [43] using bovine serum albumin as the standard.

2.6. Serum anti-SRBC IgM analysis by ELISA

Rats were bled by tail nicking six days after immunization with SRBC. Blood samples were allowed to clot at room temperature for 1–1.5 h. Serum was separated by centrifugation at 850 × g for 20 min. The concentration of anti-SRBC IgM was measured in the rat serum samples by direct non-competitive ELISA [44], which involved the use of detergent-solubilized SRBC ghosts immobilized onto microtiter wells and horseradish peroxidase-conjugated antibody for detection.

Briefly, the SRBC membrane preparation was diluted to a protein concentration of 0.67 g/mL in 0.1 M sodium carbonate-bicarbonate buffer, pH 9.5, and added (150 μL/per well) to a Nunc-Immuno microtiter plate (A/S Nunc, Roskilde, Denmark), followed by overnight incubation at 4 °C. The next day, the contents of the microtiter plate were discarded and each well was washed three times with

150 μL of 0.01% Tween 20 in distilled water for 1–2 min per wash. Wells were then filled (150 μL/well) with 0.05% Tween 20 in phosphate-buffered saline (PBS, containing 0.137 M NaCl, 2.6 mM KCl, 8.1 mM sodium phosphate, and 0.15 M potassium phosphate, pH 7.4) and incubated for 2 h at 37 °C in a shaking water bath, followed by three wash steps. Meanwhile, rat serum samples were diluted with 0.01% Tween 20 in distilled water. Typically, ten serial (two-fold) dilutions of each serum sample were used. A 150-μL aliquot of each dilution of rat serum was added to a SRBC-coated well in duplicate. The microtiter plates were incubated for 2 h at 37 °C in a shaking water bath. The wells were washed as before and 150 μL of horseradish peroxidase-conjugated mouse anti-rat IgM (500 ng/mL) was then added to each well. The microtiter plates were then incubated for 1 h at 37 °C with shaking. Each well was washed thoroughly and 150 μL/well of substrate solution consisting of 92.2 mM *o*-phenylenediamine and 0.01% hydrogen peroxide in 0.1 M sodium citrate buffer, pH 5.0, was added. The reaction was allowed to proceed for 5 to 10 min and was stopped by addition of 40 μL/well of 4 M sulfuric acid. Absorbance at 490 nm was measured using a Bio-Tek plate reader (Bio-Tek Instruments, Winooski, VT). Serum anti-SRBC IgM concentrations, assessed as the serum IgM titer yielding an absorbance value of 1, were determined from extrapolations of plots of absorbance values versus the anti-SRBC IgM titer for each set of serial dilutions. Each serum sample was assayed at least twice.

2.7. Determination of serum hormone concentrations by radioimmunoassay

Rat trunk blood was collected at the time of death, allowed to clot at room temperature for 1–1.5 h and serum was separated by centrifugation at 850 × g for 20 min. Serum T4 and testosterone concentrations were measured using antibody-coated tube RIA kits supplied by ICN Biomedicals Canada Ltd. (ICN Biomedicals Canada Ltd., St-Laurent, Quebec, Canada). Luteinizing hormone (LH) and thyroid-stimulating hormone (TSH) concentrations were measured using double antibody RIA with reagents provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) [25].

2.8. Testosterone hydroxylase assay

Testosterone hydroxylase activities were determined using a high performance liquid chromatography assay as reported previously [39,45]. Reactions were allowed to proceed for 5 min at 37 °C. Testosterone and metabolites were analyzed using a Shimadzu LC-600/LC-9A binary gradient HPLC system (Shimadzu Scientific Instruments, Columbia, MD, USA). Metabolites were resolved at 40 °C on a Supelcosil LC-18 (Supleco, Bellefonte, PA) octyldecylsilane reverse phase column (5 μm particle size, 15 cm × 46 mm id). Gradient elution of the HPLC column was achieved using a mobile phase consisting of 100% solvent A (methanol:water: acetonitrile, 36:64:1) for the first 10 min, followed by a linear gradient to 100% solvent B (methanol: water: acetonitrile, 80:18:2) from 10 to 29 min, then 100% solvent B from 29 to 31 min, followed by a return to 100% solvent A from 31 to 32 min, and equilibration with 100% solvent A for a further 2 min. Total flow rate for the analysis was 2 mL/min. Assay conditions were selected to ensure that substrate and cofactor concentrations were saturating and that product formation was first order with respect to incubation time and microsomal protein concentration.

2.9. Immunoblot analysis of CYP proteins

Hepatic microsomal proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis **(**SDS-PAGE) according to the procedure of Laemmli [46] as described previously [39]. Proteins separated by SDS-PAGE were transferred electrophoretically (0.4 A for 2 h at 4 °C) onto nitrocellulose membranes according to the procedure of Towbin et al. [47]. The membranes were incubated for 2 h at 37 °C with rabbit anti-rat CYP2B1 IgG at 2 μg/mL or mouse anti-rat CYP1A1 IgG at 1 μg/mL. Membranes were then washed and incubated for 2 h at 37 °C with alkaline phosphatase-conjugated goat $F(ab')_2$ anti-rabbit IgG or anti-mouse IgG (Tago BioSource International, Camarillo, CA) at a dilution of 1:3000. Membranes were washed again and visualization of the protein bands was achieved by incubation with a substrate solution consisting of 0.01% *p*-nitro-blue tetrazolium chloride and 0.05% 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M Tris-HCl buffer containing 0.5 mM $MgCl_2$, pH 9.5. Assay conditions were optimized to ensure that color development did not proceed beyond the linear range of the phosphatase reaction. The staining intensities of the protein bands were measured with a pdi 420oe densitometer (PDI, Inc., New York, NY) equipped with an AGFA Arcus II scanner using the pdi Quantity One® 3.0 software. A single concentration of the appropriate purified CYP protein was included on each blot as an internal standard. The amount of immunoreactive protein was determined from the integrated intensity of the stained band to that of the internal standard. Values of the ratio of the integrated intensities were converted into pmole quantities of CYP1A1, CYP1A2, CYP2B1, or CYP2B2 by means of calibration curves generated with purified rat CYP standards.

2.10. Statistical analysis

Parametric tests were used when the data passed the normality test. Non-parametric tests were used when the data failed to pass the normality and equal variance tests. Differences between mean values of treatment groups were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test (parametric) or the Kruskal-Wallis test (non-parametric), using Instat software Ver. 3.0 (GraphPad Software, Inc., San Diego, CA). A p value less than 0.05 was considered to be statistically significant.

3. Results

Administration of Aroclor 1260 to adult male rats for 10 days at dosages ranging from 25 μg/kg/day to 156 mg/kg/day did not affect body weight, except for the highest dosage group, which had a final mean body weight that was 14% lower than that of the control group (Table 1). Aroclor 1260 elicited a dosage-dependent increase in liver weight and the relative liver weight of the highest dosage group was almost twice that of the control group due to increased liver weight combined with decreased body weight.

To assess effects on the immune system, a functional endpoint associated with the immune response, namely the ability of rats to mount an immune response when challenged with a foreign antigen (i.e. SRBC), and a morphological endpoint, thymus weight, were examined. Humoral immunity was suppressed as shown by a dose-dependent decrease in anti-SRBC IgM titer at dosages of 6.25 mg/kg/day and greater (Table 2). For animals in the highest dosage group, the anti-SRBC IgM titer was 18% that of the control group. Treatment with Aroclor 1260 did not alter thymus weight (Table 2).

Table 1. Body and liver weights of adult male rats treated with Aroclor 1260.

Dosage (mg/kg/day)	Final body weight (g)	Weight gain (g)	Liver weight (g)	Relative liver weight (% of final body weight)
Corn oil	324 ± 9	78 ± 7	14.0 ± 0.5	4.3 ± 0.1
0.025	333 ± 5	81 ± 4	15.5 ± 0.4	4.7 ± 0.1
0.125	333 ± 5	84 ± 6	15.0 ± 0.5	4.5 ± 0.1
0.625	323 ± 9	81 ± 5	14.8 ± 0.7	4.6 ± 0.1
1.25	321 ± 8	82 ± 3	15.1 ± 0.6	4.7 ± 0.1
3.13	336 ± 9	85 ± 8	17.2 ± 0.8 [a]	5.1 ± 0.1 [a]
6.25	321 ± 4	81 ± 6	18.2 ± 0.5 [a,b]	5.7 ± 0.1 [b]
15.6	324 ± 5	81 ± 3	19.7 ± 0.4 [a,b]	6.1 ± 0.2 [b,c]
31.3	316 ± 3	76 ± 4	20.3 ± 0.7 [b,c]	6.4 ± 0.2 [c]
78.1	301 ± 6	67 ± 6	23.0 ± 0.9 [c,d]	7.6 ± 0.2 [d]
156	280 ± 22 [a]	27 ± 18 [a]	24.1 ± 0.9 [d]	8.8 ± 0.5 [e]

Data are expressed as means ± SE of 6 rats per treatment group, except for the 156 mg/kg/day dosage group where $n = 5$. Adult male rats were treated with Aroclor 1260 in corn oil by oral gavage, once daily for 10 consecutive days at the dosages indicated, and were killed 2 days after the last treatment. The vehicle-treated (control) group was treated with corn oil only.

Superscript letters, a-e, indicate that the mean value of the Aroclor 1260-treated group is significantly different ($p < 0.05$) from the corresponding value of the control (corn oil-treated) group. Treatment groups labelled with different letters are also significantly different ($p < 0.05$) from each other, whereas groups labelled with the same letters are not statistically different from each other. Mean values lacking a superscript symbol are not significantly different from the control group.

Table 2. Thymus weights and humoral immune response of adult male rats treated with Aroclor 1260.

Dosage (mg/kg/day)	Anti-SRBC IgM titer	Thymus weight (g)
Corn oil	483 ± 80	0.47 ± 0.02
0.025	449 ± 121	0.44 ± 0.04
0.125	583 ± 105	0.48 ± 0.02
0.625	403 ± 75	0.53 ± 0.04
1.25	283 ± 46	0.57 ± 0.03
3.13	244 ± 26	0.57 ± 0.05
6.25	161 ± 24 [a]	0.54 ± 0.03
15.6	148 ± 27 [a]	0.45 ± 0.05
31.3	135 ± 20 [a]	0.46 ± 0.03
78.1	112 ± 14 [a]	0.49 ± 0.04
156	86 ± 13 [a]	0.37 ± 0.06

Data are expressed as the means ± SE for the same treatment groups as in Table 1. Rats were injected with SRBC (2×10^8 cells) 4 days after the start of Aroclor 1260 treatment and blood for anti-SRBC IgM determination

was collected 6 days later. Values are expressed as the anti-SRBC IgM titer required to yield an absorbance value of 1 as described in the Materials and Methods section.

The superscript letter, a, indicates that the mean value of the Aroclor 1260-treated group is significantly different ($p < 0.05$) from the corresponding value of the control (corn oil-treated) group. Treatment groups labelled with the same letters are not statistically different from each other. Mean values lacking a superscript symbol are not significantly different from the control group.

The effects of Aroclor 1260 treatment on endocrine endpoints are summarized in Table 3. Serum T4 concentrations were decreased by approximately 60% to 80% following treatment with Aroclor 1260 at dosages of 6.25 mg/kg/day and greater. In contrast, serum TSH levels were only significantly different from that of the control group for rats treated with Aroclor 1260 at a dosage of 15.6 mg/kg/day. Aroclor 1260 treatment had no effect on serum testosterone or LH concentrations. Moreover, testes, seminal vesicles, and ventral prostate weights were unchanged at all dosages of Aroclor 1260.

Aroclor 1260 altered testosterone biotransformation in liver microsomes (Table 4). Formation of 2α-hydroxytestosterone, which is a major metabolite of male rats, was decreased in a dosage-dependent manner following treatment with Aroclor 1260 at dosages of 6.25 mg/kg/day and greater. For the highest dosage group, the testosterone 2α-hydroxylase activity was only 4% that of the control group (Table 4). In comparison, testosterone 2β-, 6β-, and 7α-hydroxylase activities were increased by 2 to 5 times following treatment with the largest dosage of Aroclor 1260 (i.e. 156 mg/kg/day), while testosterone 16β-hydroxylase activity was increased 42-fold following treatment with Aroclor 1260 at a dosage of 1.25 mg/kg/day and 100-fold after treatment with 6.25 mg/kg/day. Interestingly, a decline in testosterone 16β-hydroxylase activity was observed with larger dosages signifying that Aroclor 1260 treatment had a biphasic effect on this activity. Hepatic microsomal testosterone 16α-hydroxylase activity also displayed a biphasic response with a slight increase in activity at dosages of 3.13 to 31.3 mg/kg/day and a decline at higher dosages. A similar trend was observed with androstenedione formation.

Quantitative analysis of hepatic CYP1A1, CYP1A2, CYP2B1 and CYP2B2 protein levels showed that CYP2B enzymes were affected by Aroclor 1260 to a greater extent than CYP1A enzymes. As shown in Table 5, the total hepatic CYP content was increased by Aroclor 1260 to a level that was almost three times greater in rats treated with the largest dosage relative to the control group. Hepatic CYP2B1 protein levels were increased in a dosage dependent manner by Aroclor 1260 treatment. The CYP2B1 content of hepatic microsomes from rats treated with the largest dosage was approximately 230 times greater than those of the control group. Hepatic CYP2B2 protein levels were similarly induced by Aroclor 1260 treatment. Hepatic CYP1A1 protein levels were below the limit of quantification for rats treated with Aroclor 1260 at dosages up to 1.25 mg/kg/day, but were easily measurable in hepatic microsomes from rats treated with 3.13 mg/kg/day and were further increased at greater dosages. Hepatic CYP1A2 was measurable in the control group and CYP1A2 levels were increased 12-fold for rats treated with the highest dosage of Aroclor 1260. Interestingly, the sum of CYP1A and CYP2B enzyme levels (listed in the table) add up to more than 100% of the total hepatic CYP content for the high dosage treatment groups. A major reason is the difference in assays used to measure total CYP content versus individual CYP enzyme levels. Immunoquantification by densitometric analysis of immunoblots, although useful for measuring relative protein expression, can overestimate protein levels, whereas analysis of the carbon monoxide difference spectrum can underestimate total CYP content.

Table 3. Serum hormone concentrations and reproductive organ weights of adult male rats treated with Aroclor 1260.

Dosage (mg/kg/day)	T4 concentration (ng/mL)	TSH concentration (ng/mL)	Testosterone concentration (ng/mL)	LH concentration (ng/mL)	Testes weight (g)	Seminal vesicle weight (g)	Ventral prostate weight (g)
Corn oil	45.8 ± 3.2	2.71 ± 0.83	5.79 ± 1.56	0.65 ± 0.06	3.16 ± 0.12	0.87 ± 0.05	0.27 ± 0.01
0.025	50.6 ± 3.3	3.44 ± 0.70	7.84 ± 2.94	0.79 ± 0.22	3.49 ± 0.12	1.24 ± 0.11	0.31 ± 0.03
0.125	47.3 ± 4.5	2.65 ± 0.60	5.42 ± 0.76	0.85 ± 0.07	3.47 ± 0.09	0.99 ± 0.07	0.29 ± 0.02
0.625	53.3 ± 1.5	3.12 ± 0.80 (n = 4)	6.57 ± 1.27	0.86 ± 0.16 (n = 4)	3.22 ± 0.11	0.93 ± 0.04	0.25 ± 0.01
1.25	41.5 ± 1.9	3.33 ± 0.84 (n = 5)	3.95 ± 0.67	0.82 ± 0.09	3.19 ± 0.06	0.92 ± 0.08	0.28 ± 0.03
3.13	34.9 ± 2.4	6.32 ± 1.03	4.33 ± 0.68	0.64 ± 0.10	3.15 ± 0.08	0.99 ± 0.06	0.29 ± 0.02
6.25	17.7 ± 1.6 [a]	5.73 ± 1.21	4.74 ± 0.85	0.59 ± 0.21	3.34 ± 0.11	0.84 ± 0.05	0.26 ± 0.03
15.6	17.6 ± 1.6 [a]	9.75 ± 1.86 [a]	3.55 ± 0.40	0.59 ± 0.09	3.22 ± 0.04	0.94 ± 0.09	0.30 ± 0.03
31.3	15.3 ± 1.1 [a]	6.39 ± 0.85 (n = 5)	6.20 ± 1.57	0.55 ± 0.08	3.21 ± 0.10	0.97 ± 0.04	0.29 ± 0.01
78.1	11.5 ± 1.5 [a]	7.27 ± 2.17 (n = 5)	5.40 ± 0.89	1.38 ± 0.93 (n = 3)	3.18 ± 0.17	0.76 ± 0.06	0.22 ± 0.02
156	10.3 ± 1.2 [a]	7.22 ± 2.97 (n = 3)	4.88 ± 2.15	0.68 ± 0.18 (n = 3)	3.37 ± 0.15	0.68 ± 0.14	0.22 ± 0.02

Data are expressed as means ± SE for the same treatment groups as in Table 1, with the following exception. Fewer serum samples were analyzed for TSH and LH concentrations for some dosage groups, as indicated in the table. Hormone concentrations were measured by RIA as indicated in the Materials and Methods section in serum prepared from trunk blood collected upon decapitation.

The superscript letter, a, indicates that the mean value of the Aroclor 1260-treated group is significantly different ($p < 0.05$) from the corresponding value of the control (corn oil-treated) group. Treatment groups labelled with the same letter are not statistically different from each other. Mean values lacking a superscript symbol are not significantly different from the control group.

Table 4. Hepatic microsomal testosterone hydroxylase activities of adult male rats treated with Aroclor 1260.

Dosage (mg/kg/day)	Testosterone metabolite formed (pmole/min/mg protein)						
	2α	2β	6β	7α	16α	16β	androstenedione
Corn oil	2590 ± 330	143 ± 18	1650 ± 230	236 ± 23	2770 ± 375	33 ± 16	1750 ± 164
0.025	2480 ± 191	152 ± 13	1830 ± 155	233 ± 10	2670 ± 235	35 ± 17	1790 ± 148
0.125	2420 ± 243	159 ± 18	1820 ± 161	258 ± 20	2600 ± 295	77 ± 17	1740 ± 170
0.625	2340 ± 141	196 ± 21	1920 ± 205	368 ± 21	3180 ± 288	637 ± 104	2380 ± 188
1.25	2100 ± 157	237 ± 19	2260 ± 197	347 ± 31	3840 ± 388	1400 ± 271 [a]	3300 ± 305 [a]
3.13	1850 ± 58	295 ± 23	2540 ± 170	347 ± 48	4600 ± 217 [a]	2290 ± 150 [a,b]	4290 ± 169 [a,c]
6.25	1430 ± 106 [a]	562 ± 56 [a]	4040 ± 398 [a,b]	799 ± 81 [a]	5570 ± 185 [a]	3360 ± 113 [b,c]	5630 ± 217 [b]
15.6	1120 ± 176 [a]	504 ± 29 [a]	3650 ± 238 [a,b]	649 ± 87 [b]	4830 ± 242 [a]	3020 ± 98 [b,c]	4920 ± 121 [b,c]
31.3	962 ± 137 [a,b]	598 ± 57 [a]	4180 ± 408 [a]	1080 ± 145 [c]	4390 ± 497	2740 ± 287 [b,c,d]	4820 ± 395 [b]
78.1	573 ± 90 [a,b]	570 ± 32 [a]	4030 ± 243 [a,b]	1000 ± 93 [c]	3800 ± 467	2730 ± 36 [c,d]	4510 ± 363 [b]
156	110 ± 59 [b]	614 ± 48 [a]	3590 ± 149 [a]	1120 ± 147 [c]	2700 ± 479	2030 ± 330 [d]	3680 ± 359 [a,b]

Data are expressed as means ± SE for the same treatment groups as in Table 1. Testosterone hydroxylase activities of hepatic microsomes prepared from individual rats were measured using an HPLC-based assay as indicated in the Materials and Methods section.

Superscript letters, a–d, indicate that the mean value of the Aroclor 1260-treated group is significantly different ($p < 0.05$) from the corresponding value of the control (corn oil-treated) group. Treatment groups labelled with different letters are also significantly different ($p < 0.05$) from each other, whereas groups labelled with the same letters are not statistically different from each other. Mean values lacking a superscript symbol are not significantly different from the control group.

Table 5. Hepatic microsomal CYP1A1, CYP1A2, CYP2B1, CYP2B2 and total CYP content of adult male rats treated with Aroclor 1260.

Dosage (mg/kg/day)	Total CYP (nmol/mg protein)	CYP1A1 content (pmol/mg protein)	CYP1A2 content (pmol/mg protein)	CYP2B1 content (pmol/mg protein)	CYP2B2 content (pmol/mg protein)
Corn oil	0.70 ± 0.02	< 0.4	3 ± 0.7	6 ± 2	4 ± 1
0.025	0.81 ± 0.05	< 0.4	3 ± 0.7	11 ± 6	10 ± 6
0.125	0.74 ± 0.07	< 0.4	3 ± 0.5	19 ± 9	16 ± 7
0.625	0.91 ± 0.06	< 0.4	3 ± 0.6	75 ± 10	67 ± 9
1.25	1.13 ± 0.08	< 0.4	3 ± 0.7	189 ± 44	120 ± 26
3.13	1.53 ± 0.06 [a]	11 ± 3	4 ± 0.7	637 ± 93 [a]	360 ± 57
6.25	1.85 ± 0.09 [a,b]	22 ± 2	13 ± 2	702 ± 71 [a]	575 ± 90 [a]
15.6	1.66 ± 0.10 [a,b]	31 ± 3 [a]	14 ± 2	952 ± 139 [a,b]	603 ± 101 [a]
31.3	1.94 ± 0.14 [a,b]	60 ± 6 [b]	23 ± 4 [a]	1389 ± 148 [b,c]	809 ± 141 [a]
78.1	1.76 ± 0.11 [a,b]	64 ± 9 [b,c]	32 ± 7 [a]	1336 ± 182 [b,c]	798 ± 105 [a]
156	2.07 ± 0.21 [b]	93 ± 15 [c]	36 ± 4 [a]	1430 ± 298 [b,c]	929 ± 137 [a]

Data are expressed as means ± SE for the same treatment groups as in Table 1. Numbers in parentheses denote individual CYP enzyme levels as a percentage of total spectrally-determined CYP. CYP1A1 was either undetectable or below the limit of quantification (< 0.4 pmol/mg microsomal protein) for some dosage groups, as indicated in the table.

Superscript letters, a–c, indicate that the mean value of the Aroclor 1260-treated group is significantly different ($p < 0.05$) from the corresponding value of the control (corn oil-treated) group. Treatment groups labelled with different letters are also significantly different ($p < 0.05$) from each other, whereas groups labelled with the same letters are not statistically different from each other. Mean values lacking a superscript symbol are not significantly different from the control group.

To determine the relative sensitivity of immune, endocrine, and hepatic responses following Aroclor 1260 exposure, experimentally determined NOAEL levels and LOAEL levels were compared (Table 6). The results indicate that increases in hepatic testosterone 16β-hydroxylase activity and androstenedione formation (i.e. testosterone 17-oxidase/dehydrogenase activity) were the most sensitive responses to Aroclor 1260 exposure, followed closely by increased liver weight, increased testosterone 16α-hydroxylase activity and induction of hepatic CYP2B1 protein and total hepatic CYP content. In contrast, endocrine endpoints including serum LH and testosterone concentrations, as well as reproductive organ weights, were the least sensitive responses to Aroclor 1260 exposure.

Table 6. No Observable Adverse Effect Levels (NOAEL) and Lowest Observable Adverse Effect Levels (LOAEL) measured in adult male rats treated with Aroclor 1260.

Response	NOAEL (mg/kg/day)	LOAEL (mg/kg/day)
Hepatic testosterone 16β-hydroxylase activity	0.625	1.25
Hepatic androstenedione formation	0.625	1.25
Liver weight	1.25	3.13
Hepatic testosterone 16α-hydroxylase activity	1.25	3.13
Total hepatic CYP content	1.25	3.13
Hepatic CYP2B1 content	1.25	3.13
Serum anti-SRBC IgM titer	3.13	6.25
Serum T4 concentration	3.13	6.25
Hepatic testosterone 2α-, 2β-, 6β-, and 7α-hydroxylase activities	3.13	6.25
Hepatic CYP2B2 content	3.13	6.25
Hepatic CYP1A1 content	6.25	15.6
Hepatic CYP1A2 content	15.6	31.3
Body weight	78.1	156
Thymus weight	156	N/A
Serum LH and testosterone concentrations	156	N/A
Testes, seminal vesicle, and ventral prostate weights	156	N/A

The NOAEL is defined as the highest exposure level at which the experimentally determined response of the treated group was not significantly different from that of the control group. The LOAEL is defined as the lowest exposure level at which the experimentally determined response of the treated group was significantly different from that of the control (corn oil-treated) group.

4. Discussion

Of the many previous animal studies of PCB toxicity that provided data for NOAEL or LOAEL determinations, most focused on reproductive and developmental toxicity and involved treatment with single PCB congeners or with Aroclor 1254 [48]. Less information is available regarding the responses produced by Aroclor 1260, which differs from Aroclor 1254 in containing a lower percentage of coplanar PCB congeners and a larger percentage of highly chlorinated and di-*ortho*-substituted PCB congeners such as PCB 138, PCB 153 and PCB 180 [11,37]. Aroclor 1260 can be considered to be more representative of the PCBs found in biota because highly chlorinated and *ortho*-substituted congeners represent a major fraction of PCBs found in environmental samples and tissues of exposed animals [11,49].

Liver was the only organ, of those examined, that was altered by Aroclor 1260 exposure. Increased liver weight is a relatively common response of PCB treatment in rats, especially at large

dosages [36,50,51]. In a comprehensive carcinogenicity study conducted with Aroclors 1016, 1242, 1254 and 1260, Mayes and co-workers noted that liver weight was increased in female but not male Sprague-Dawley rats administered Aroclor 1254 or 1260 in the diet at concentrations of 25, 50 or 100 ppm (approximately equivalent to dosages of 1, 3 or 6 mg/kg/day) for 24 months [50]. In comparison, Ngui and Bandiera reported increased liver weight in male Long Evans rats that had been treated with Aroclor 1260 by i.p. injection at dosages of 20 or 50, but not at dosages of 10 or 5 mg/kg/day for 7 days [36]. Van der Plas and co-workers [51] observed that liver weight was increased after treatment of female Sprague-Dawley rats with Aroclor 1260 by s.c. injection at a dosage of 10 mg/kg/week for 20 weeks. Taken together, the three studies suggest that adult female rats are somewhat more sensitive to PCB-elicited liver enlargement than male rats. In the present study, the LOAEL for liver enlargement in male rats was 3.13 mg/kg/day. Although this finding is not consistent with results of the studies by Ngui and Bandiera [36] and Mayes and co-workers [50], comparison across studies using different treatment protocols (e.g. routes of administration and exposure times) can be problematic because of differences in absorption and bioavailability that are likely to influence the NOAEL and LOAEL.

The ability of Aroclor 1260-treated rats to mount a primary antibody response to a challenge with sheep red blood cells was assessed by the anti-SRBC IgM titer, which is a frequently used endpoint for evaluating the potential immunotoxicity of a xenobiotic. Aroclor 1260 treatment decreased anti-SRBC IgM titer with a LOAEL of 6.25 mg/kg/day. An earlier study reported that acute treatment of B6C3F1 female mice with Aroclor 1260 at dosages of 50 to 1000 mg/kg resulted in dose-dependent reduction of humoral immunity as measured using serum anti-trinitrophenyl-lipopolysaccharide IgM titer and the splenic plaque-forming cell assay [30]. Results of that study indicated that Aroclors 1242, 1248, 1254 and 1260 elicited comparable levels of immunotoxicity, despite wide differences among the technical mixtures in terms of toxic equivalents and potency as inducers of hepatic microsomal EROD activity. The mechanism by which PCBs suppress humoral immunity is unclear. Earlier studies found evidence that immune suppression induced by PCBs and related compounds was mediated by the Ah receptor [52,53]. However, many di-*ortho* substituted PCBs with low affinity for the Ah receptor were found to be as potent as coplanar and mono-*ortho* congeners in immunotoxicity assays [30,54], suggesting that PCBs can also suppress humoral immunity through an Ah receptor-independent mechanism.

The thymus gland, which is a critical component of the T-cell dependent antibody response, was also measured in the current study as an immunotoxic endpoint. Thymus weight was not affected in our study. Harris and coworkers [55] had previously reported that thymus weight was unchanged in immature male Wistar rats after a single i.p. injection of Aroclor 1260 at dosages ranging from 10 to 2000 mg/kg. Similarly, Van der Plas and co-workers [51] noted that thymus weight was unchanged after chronic treatment of female Sprague Dawley rats with Aroclor 1260 at a dosage of 10 mg/kg/week by s.c. injection for 20 weeks. In contrast, thymic atrophy was observed for female New Zealand rabbits treated s.c. with Aroclor 1260 at a dosage of 120 mg/kg/day for 28 days [56,57], suggesting that the thymus gland might be more sensitive to PCB treatment in some experimental animals.

The thyroid and, in particular, thyroid hormone homeostasis, have been proposed as endocrine targets for PCB toxicity [18,23,58]. Treatment with Aroclor 1260 treatment elicited a decrease in circulating T4 concentration with a LOAEL of 6.25 mg/kg/day. Serum TSH concentration was increased in rats that had been treated at 15.6 mg/kg/day and was not significantly increased at higher dosages of Aroclor 1260. Our results are in general agreement with earlier studies showing that treatment of rats

with coplanar and non-coplanar PCB congeners and mixtures such as Aroclor 1254 decreased serum T4 levels, without altering serum T3 or TSH levels [22,59]. By reducing circulating levels of T4 levels, PCBs can disrupt thyroid hormone action. One of the mechanisms by which PCBs can alter serum T4 concentrations is increased hepatic metabolism of T4 through induction of hepatic UDP-glucuronosyl transferase (UGT) enzymes, especially UGT1A1 and UGT1A6, which leads to increased biliary excretion [60,61]. A second mechanism is the displacement of T4 from transthyretin by the binding of PCBs to this thyroxine plasma transport protein [62]. By reducing the carrying capacity of the blood for T4, the hormonal activity is decreased, and clearance and elimination of T4 are facilitated.

Although previous *in vitro* and animal studies suggested that PCBs are weakly estrogenic [28], no changes in serum testosterone and LH levels, and testis, ventral prostate and seminal vesicle weights were detected for rats in the present study following treatment with Aroclor 1260. Alterations in the extent and in the pattern of testosterone biotransformation were readily apparent, however, following Aroclor 1260 treatment. The rates of formation of minor metabolites such as 2β-, 7α-, and especially 16α-hydroxytestosterone were increased, while formation of a major metabolite, namely 2α-hydroxytestosterone, was markedly decreased by Aroclor 1260 exposure. It is well established that testosterone is oxidized by multiple hepatic CYP enzymes in a regio-selective manner. For example, testosterone 2α-, 2β- (and 6β-), 7α-, and 16β-hydroxylase activities are catalyzed almost exclusively by CYP2C11, CYP3A1, CYP2A1, and CYP2B1, respectively, while CYP2C11 and CYP2B enzymes contribute to testosterone 16α-hydroxylase activity and androstenedione formation [63,64]. Hence, the increase in testosterone 2β- and 7α-hydroxylase activities, and the decrease in testosterone 2α-hydroxylase activity, observed after treatment with Aroclor 1260 could be attributed to increased expression of CYP3A and CYP2A1 enzymes and reduced expression of CYP2C11. In support, Ngui and Bandiera [36] reported that hepatic protein levels of CYP3A and CYP2A1 enzymes were increased and CYP2C11 protein levels were decreased in rats treated with Aroclor 1260 at dosages of 5 to 50 mg/kg/day. Biphasic dose-responses were observed for testosterone 16α- and 16β-hydroxylase activities and androstenedione formation. The biphasic appearance of the dose-responses for testosterone 16α-hydroxylase activity and androstenedione formation can be similarly explained by induction of CYP2B and suppression of CYP2C11 enzymes. The decline in testosterone 16β-hydroxylase activity at the highest dosages of Aroclor 1260 is not easily explained because immunoquantitative analysis of CYP2B enzyme expression indicated that hepatic CYP2B1 and CYP2B2 protein levels were increased and did not decline after treatment with the higher dosages of Aroclor 1260. The two CYP2B enzymes were more highly induced by Aroclor 1260 than CYP1A enzymes, and of the two CYP2B enzymes, induction of CYP2B1 (LOAEL = 3.13 mg/kg/day) was a more sensitive endpoint than induction of CYP2B2.

In conclusion, the results indicate that the major target organ for Aroclor 1260 in adult rats is the liver and that among the endpoints assessed, hepatic biochemical endpoints, specifically CYP2B-mediated testosterone hydroxylase activities, were a more sensitive response to Aroclor 1260 exposure than immune or endocrine endpoints. Toxicity analysis of a complex mixture such as Aroclor 1260 should not imply that the individual PCB congeners comprising the mixture elicit all of the same effects. Some congeners elicit few toxic responses whereas other PCB congeners might produce more selective responses compared with those of the mixture. Additional responses elicited by PCBs, but not measured in the current study, include changes in serum cholesterol levels, impaired neurological function and disruption of intracellular calcium homeostasis and ryanodine receptor activity [65,66,67],

signifying that the total health risk associated with Aroclor 1260 exposure is determined by multiple biochemical and toxic effects impacting several organ systems.

5. Conclusion

Toxic and biochemical endpoints of exposure to Aroclor 1260 were evaluated in a dose-response study involving adult male rats. Arolcor 1260 is a complex commercial mixture containing a relatively high percentage of highly chlorinated and di-*ortho* substituted PCB congeners that currently predominate in environmental and biological samples, including human tissue samples. Endpoints examined included anti-SRBC IgM titer, thyroid hormone levels, hepatic microsomal CYP1A and CYP2B enzyme content and testosterone hydroxylase activities and corresponding NOAEL and LOAEL were determined. Comparison of NOAEL and LOAEL values indicated that the more sensitive endpoints affected by Aroclor 1260 were increased testosterone 16β- and 16α-hydroxylase activity and androstenedione formation, increased liver weight, and increased CYP2B protein content. Reduced serum thyroxine levels and anti-SRBC IgM titer, which represent alterations of an endocrine and immune system, respectively, were less sensitive responses, as reflected by slightly larger LOAEL values. Although chronic low-level PCB exposures remain a significant health concern, the results suggest that even the most sensitive responses detected in the study are unlikely to occur as long as human exposure to PCBs from environmental sources remains below the tolerable daily intake value of 20 ng/kg/day set by the World Health Organization.

Conflict of interest

The authors declare no conflicts of interest in the study.

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