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Postnatal exposure of 2,2′,3,3′,4,6′-hexachlorobiphenyl and 2,2′,3,4′,5′,6-hexachlorobiphenyl on sperm function and hormone levels in adult rats

Ping-Chi Hsu [a], Mei-Hui Li [b], Yueliang Leon Guo [c,*]

[a] *Department of Safety, Health and Environmental Engineering, National Kaohsiung First University of Science and Technology, 1, University Road, Yuanchau, Kaohsiung, Taiwan, ROC*
[b] *Environmental Toxicology Laboratory, Department of Geography, National Taiwan University, 1, Section 4, Roosevelt Road, Taipei 106, Taiwan, ROC*
[c] *Department of Environmental and Occupational Health, National Cheng Kung University Medical College, 61 Shiao Tong Road, Tainan 70428, Taiwan, ROC*

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Abstract

Polychlorinated biphenyls (PCBs) are known to affect reproductive system in animals and in accidentally or occupationally exposed humans. Information is lacking on effects of non-dioxin like chlorinated biphenyls (CB) congeners on male reproduction. The aim of this study is to determine whether treatment of postnatal non-dioxin like CB congeners affects sperm function and hormone levels in rats. Male Sprague–Dawley rats received either 2,2′,3,3′,4,6′-hexachlorobiphenyls (CB 132) or 2,2′,3,4′,5′,6-hexachlorobiphenyls (CB 149) by ip injection of 9.6 or 96 mg/kg at day 21. At 16 weeks, the animals were sacrificed; sperm quality and hormone levels were measured. Body weight, testis and cauda epididymis weights, sperm counts, ROS generation, acrosome reaction rate, serum thyroxine (T_4), free T_4 and testosterone (TT) concentrations were unaffected. However, treatment of CB 132 and CB 149 caused decreases in sperm motility, curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF). Serum triiodothyronine (T_3) level was significantly decreased in CB 132 9.6 mg/kg dose group compared with the controls. On the other hand, a significant decrease was found in free T_3 concentration both in 96 mg/kg of CB 132 and CB 149 groups. In summary, this study showed that CB 132 and CB 149 affects serum levels of triiodothyronine as well as sperm motility, velocity and capability of penetrating oocytes. The mechanism of action and potential effects on human warrant further investigation.

Keywords: CB 132; CB 149; Postnatal exposure; Sperm function; Triiodothyronine

* Corresponding author. Tel.: +886-6-236-5228; fax: +886-6-274-3748.
E-mail address: leonguo@mail.ncku.edu.tw (Y.L. Guo).

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1. Introduction

Polychlorinated biphenyls (PCBs) are ubiquitous environmental pollutants with 209 possible congeners possessing different numbers of chlorine atoms substituted on the biphenyl rings. Among 209 congeners, a small group of coplanar chlorobiphenyl (CB) congeners resembles 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in molecular configuration. The majority of CB congeners presented in the environment are non-coplanar. The toxicities of environmentally relevant CB congeners with at least two or more *ortho*-chlorine substitutions are still poorly understood (Hansen, 1998).

Among different *ortho*-substituted CB congeners, certain CB congeners with the labile 2,3,6-chlorine pattern on one phenyl ring, such as 2,2′,3,5′,6-pentachlorophenyl (CB 95), 2,3,3′,4′,6-pentachlorophenyl (CB 110), 2,2′,3,3′,4,6′-hexachlorophenyl (CB 132) and 2,2′,3,4′,5′,6-hexachlorophenyl (CB 149), are major components in commercial PCB mixtures (Frame et al., 1996). The 2,3,6-substituted ring is rapidly metabolized to methylsulfonyl PCBs (Noren et al., 1996), for example, both methylsulfonyl CB 132 and CB 149 metabolites can be detected in human tissues (Weistrand and Noren, 1997). Therefore, these congeners are considered to be not persistent and attracted little attention in PCB toxicity studies. More recently, there are some limited evidences suggested that 2,3,6-substitued CB congeners possessing certain toxicities that were not identified before. Most of the male reproductive problems have been observed in rodents with in utero and lactational exposure to TCDD (Mably et al., 1992) or prenatal (Faqi et al., 1998a) and/or postnatal (Huang et al., 1998; Faqi et al., 1998b) exposure to dioxin-like CB congeners. However, information concerning the effects of non-dioxin like CB congeners on male reproduction is still limited.

Both CB 132 and CB 149 are conformationally stable chiral CB congeners with the 2,3,6-chlorine pattern on one phenyl ring (Glausch et al., 1996). Considering the fact that both CB 132 and CB 149 are major components in Aroclor mixtures (Frame et al., 1996) and frequently detected in human milk (Glausch et al., 1995; Blanch et al., 1999) as well as biota (Jordan and Feeley, 1999; Reich et al., 1999; Jimenez et al., 2000; Serrano et al., 2000; Pier et al., 2002; Riedel et al., 2002), it is of importance to investigate possible endocrine-disrupting effects of these two congeners. In an attempt to further identify the toxicity of male reproduction and the hormonal pathways involved, we used Sprague–Dawley male rats as an animal model to examine the sperm function, serum steroid hormone, and serum thyroid hormone of two environmentally relevant 2,3,6-substitued CB congeners, CB 132 and CB 149.

2. Materials and methods

2.1. Overview of study design

Sprague–Dawley rats were purchased from the Animal Center of National Cheng Kung University Medical Center (Tainan, Taiwan) and were housed in a rodent vivarium under a 12-h light: 12-h dark cycle and controlled temperature. Animals were housed in plastic cages and allowed to acclimatize to their new environment for 14 days prior to initiation of treatment. Laboratory Rodent Diet 5001 (LabDiet, Richmond, IN) and water were available ad libitum. Male SD rats, 3-weeks-old, were randomly divided into control and CB-treated groups. The animals were treated with intraperitoneal injection of single dose of 9.6 or 96 mg/kg corn oil or CB 132 or CB 149 at day 21. The rationale of choosing the timing for treatment is to simulate the effect of childhood accidental PCB exposure on later reproductive system in adulthood. At 16 weeks, the animals were sacrificed and the testis and cauda epididymis were removed and weighted. Blood samples were collected by cardiac puncture for serum hormones analysis. Epididymal sperm counts, motility and velocity were measured and an epididymal sperm suspension was prepared for reactive oxygen species (ROS) assay, acrosome reaction assay, and insemination. Sperm were incubated with ova harvested from nonCB-exposed female rats, then sperm–oocyte penetration rate (SOPR) was assesses after 48 h insemination.

2.2. Chemicals

Corn oil was purchased from Sigma Chemical Company (St. Louis, MO). CB 132 and CB 149 were obtained from AccuStandard (New Haven, CT; guaranteed $>99\%$ pure). Phosphate-buffered saline (PBS) and human tubule fluid (HTF) medium were purchased from Gibco Life Technologies Ltd. (New York, USA). Ammonium dihydrogen phosphate was obtained from Merck Co. (Darmstadt, Germany). Radioimmunoassay kits for determining serum thyroid hormone were purchased from Diagnostic Systems Laboratories, Inc. (Webster, TX). All other chemicals and reagents used in this study were obtained from Sigma Chemical Company.

2.3. Sperm concentration, motility and velocity

The right cauda epididymis at termination was dissected from each male and transported to the laboratory in 1 ml 34 °C HTF buffer supplemented with 5 mg/ml bovine albumin. The cauda epididymis was removed and placed in 1 ml HTF–albumin buffer. A 1:10 dilution of spermatozoa was prepared and an epididymal sperm count done with a hemocytometer. Sperm samples were held in a Makler chamber (Sefi Medical Instruments, Israel) for sperm motility measurements. Computer-assisted sperm analysis (CASA) was obtained for velocity indices with a Hamilton Thorn Research motility analyzer (version HTM-IVOS Specification, Beverly, MA, USA) at a temperature of 37 °C. CASA was gained for sperm velocity parameters: curvilinear velocity (VSL, μm/s), average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), amplitude of lateral head displacement (ALH, μm), and beat cross frequency (BCF, Hz). Value was expressed for the performance of all sperm velocity after adjustment.

2.4. Sperm reactive oxygen species assay

A volume of 300 μl of PBS-washed rat epididymal sperm suspension at a concentration of 47×10^6 cells/ml were mixed with 100 μl of 30 mM $FeSO_4$ and then treated with 50 μl of luminol stored as a 1 mM stock solution in DMSO. The chemiluminescent signal was measured immediately with a computerdriven luminometer (Autolumat-LB 953; EG&G Berthold Co., Bad Wildbad, Germany), with the counts being integrated over a 60-s period. For each experiment, the background chemiluminescence was assessed prior to luminol addition and as found to be less than 1.7 counts/s, which was considered negligible. At the end of this period, the sperm ROS levels were measured into chemiluminescence counts per 10^6 sperm per s.

2.5. Chlortetracycline fluorescence assay

The modification of Fraser and Herod (1990) and the Oberlander et al. (1996) methods were used to detect the status of sperm acrosome reaction in rats. Fluorescence patterns of spermatozoa were analyzed with an Olympus BX-60 microscope (Tokyo) equipped with phase-contrast and epifluorescence optics. A total of 200 cells was assessed in each sample and classified as having 'unacrosomed spermatozoa,' with fluorescence over the entire head; 'acrosome-reacted spermatozoa,' with a dark head except for the tip, which retained some fluorescence.

2.6. Sperm–oocyte penetration assay

Control female Sprague–Dawley rats were superovulated at age 63–70 days weighing 330–380 g. In the morning of day 1, the rats were injected with 25 IU of PMSG. On day 3, 52 h later, the animals were injected with 25 IU of hCG. Twenty hours later, the female rats were sacrificed and the cumuli were dissected from the oviducts, collected in HTF–albumin buffer, and dissolved with 10 mg/ml hyaluronidase. After adjusting to 10×10^6 sperm/ml with HTF–albumin buffer, 10 μl of epididymal sperm suspension was added to 100 μl of HTF–albumin buffer containing 10–15 zona-intact rat ova. Each culture well of the sperm–ova preparation was overlaid with mineral oil and incubated at 37 °C in 95% air/5% CO_2. After 48 h of insemination, the number of oocytes penetrated by spermatozoa was determined by phase-contrast microscopy at $400\times$ magnification. SOPR was used to evaluate the sperm–

oocyte penetration capacity as described in the following equation:

$$\text{SOPR}(\%) = [1 - (\text{number of not penetrated oocytes}/\text{number of total oocytes})] \times 100$$

2.7. Hormone assay

The concentrations of testosterone (TT), triiodothyronine (T_3), thyroxine (T_4), free T_3 and free T_4 in the serum were measured by radioimmunoassay (International CIS radioimmunoassay Kits). Two hundreds microliter of antiserum with optimal dilution and 50 μl of tracer, circa 10 000 cpm were mixed with 300–500 μl of 1% ovalbumin in PBS containing standard hormone or sample in a 12 × 75 plastic tube. The mixture was incubated for 48 h at 4 °C before addition of the second antibody. After an additional 48-h incubation, 2 ml of ice-cold PBS were added into each assay tube and the samples were centrifuged at $1000 \times g$ at 4 °C for 30 min. The radioactivity in the precipitate was measured by automatic gamma counter. Each sample was assayed at one or two dilutions in duplicate.

2.8. Statistical analysis

Comparisons of body weights, testis weights, cauda epididymis weights, sperm counts, motility, motile velocity, sperm chemiluminescence counts, acrosome reaction rate, SOPR and serum hormone levels among CB 132 or CB 149-exposed animals and the control group were done by one-way analysis of variance (ANOVA), followed by the Tukey–Kramer honestly significantly difference (HSD) of the JMP statistical package (SAS Institute, Inc., Gary, NC).

3. Results

3.1. Body weight and organ weight

At 112 days of age, body weights, right testis, left testis and total testis weights of male rats were not affected by the treatment of CB 132 or CB 149, nor were right cauda epididymis, left cauda epididymis and total cauda epididymis weights (data not shown).

3.2. Epididymal sperm motility and velocity

There was a significant decrease in sperm motility in 9.6 mg/kg of CB 132, 96 mg/kg of CB 132 and 96 mg/kg of CB 149 groups (Fig. 1A). The value of VCL in both 96 mg/kg of CB 132 and CB 149 groups was significantly decreased compared with the controls (Fig. 1B). The value of VAP and VSL in 96 mg/kg of CB 149 group was significantly decreased compared with their controls, respectively (Fig. 1C and D). The levels of ALH and BCF in 96 mg/kg of CB 132 group were lower than those of the controls did (Fig. 1E and F).

3.3. Sperm concentrations, ROS generation, acrosome reaction and sperm–oocyte penetration rate

Comparison of the sperm counts among CB 132 treatment groups indicated that there was about 27–31% decrease in both 9.6 mg/kg and 96 mg/kg dose groups (Fig. 2A). Sperm ROS measured with chemiluminescence counts integrated over 60 s were compared and not different among rats treated with CB 132, CB 149 and the controls (Fig. 2B). There was no statistically significant difference in the status of sperm acrosome reaction among CB 132 or 149 treatment groups and the controls (Fig. 2C). The percentage of sperm–oocyte penetration was significantly reduced in 9.6 and 96 mg/kg of CB 132 groups, and 96 mg/kg of CB 149 group compared with the controls (Fig. 2D). Both CB 132 and CB 149, dose-dependently decreased the SOPR from 71 to 66% in 9.6 and 96 mg/kg of CB 132 groups, or from 78 to 64% in 9.6 and 96 mg/kg of CB 149 groups (Fig. 2D).

3.4. Serum hormone levels

There was a significant decrease on serum T_3 level in 9.6 mg/kg of CB 132 group compared with the controls (Fig. 3A). On the other hand, a significant decrease in free T_3 concentration both in 96 mg/kg of CB 132 and CB 149 groups

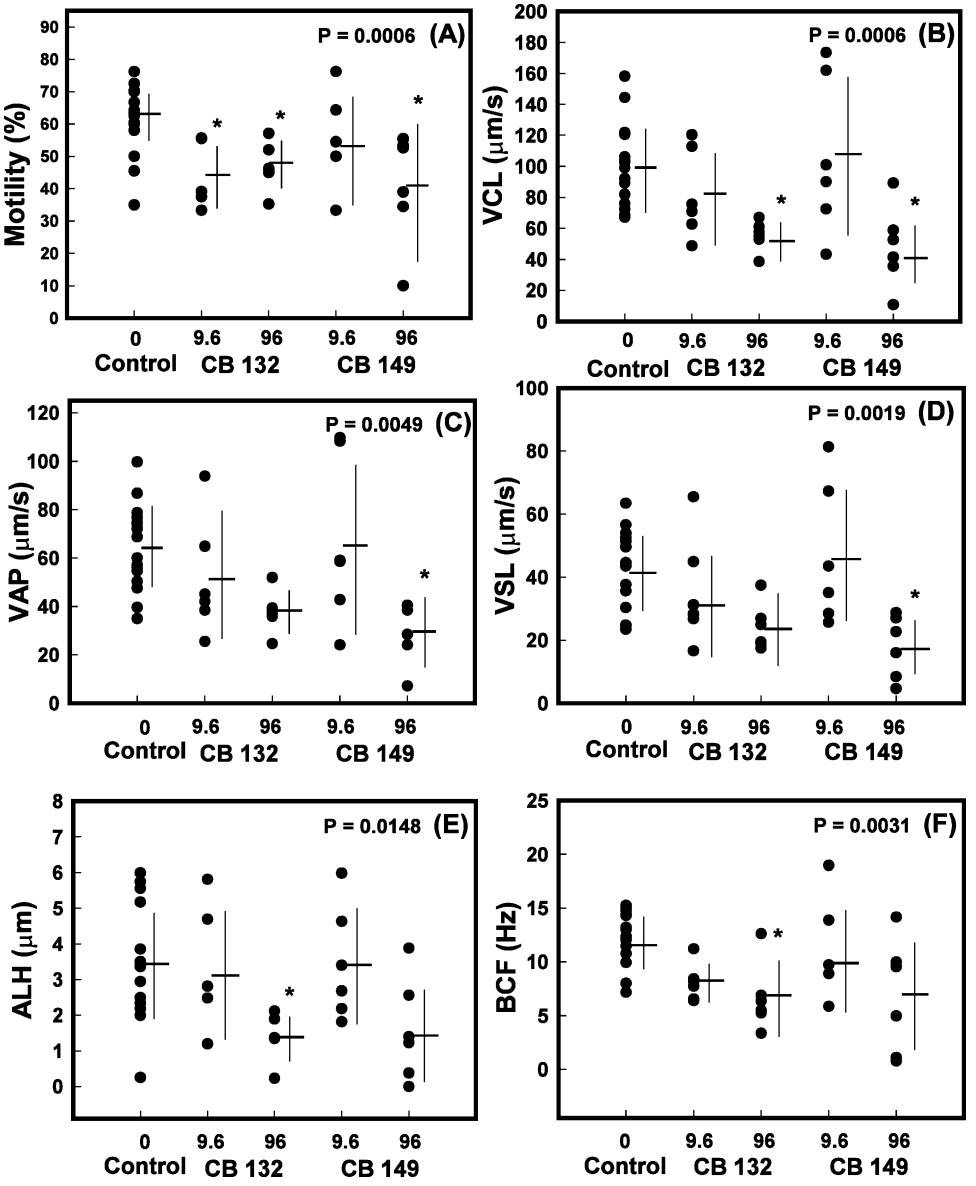

Fig. 1. Sperm motility (A), VCL (B), VAP (C), VSL (D), ALH (E), and BCF(F) were analyzed and expressed as individual values in the control group ($n = 16$), 9.6 mg/kg of CB 132 ($n = 6$), 96 mg/kg of CB 132 ($n = 6$), 9.6 mg/kg of CB 149 ($n = 6$), and 96 mg/kg of CB 149 ($n = 6$) dose groups. Because of identical values, the number of points might be less than 16 (control group) or 6 (exposed groups) in some columns. Significant difference measured by the Tukey–Kramer honestly significantly difference (HSD) was * $P < 0.05$ compared with the control group.

compared with the controls, respectively (Fig. 3B). Serum total T_4, free T_4 and TT concentrations in male rats were not significantly affected by treatment with CB 132 or 149 at 112 days of age (Fig. 3C–E). It is obvious that not all of the parameters in the 6 rats treated with CB 132 or those with CB 149 were affected, and for most of the variable analyzed these parameters stayed within the control range. However, the information indicated a beginning trend of effects.

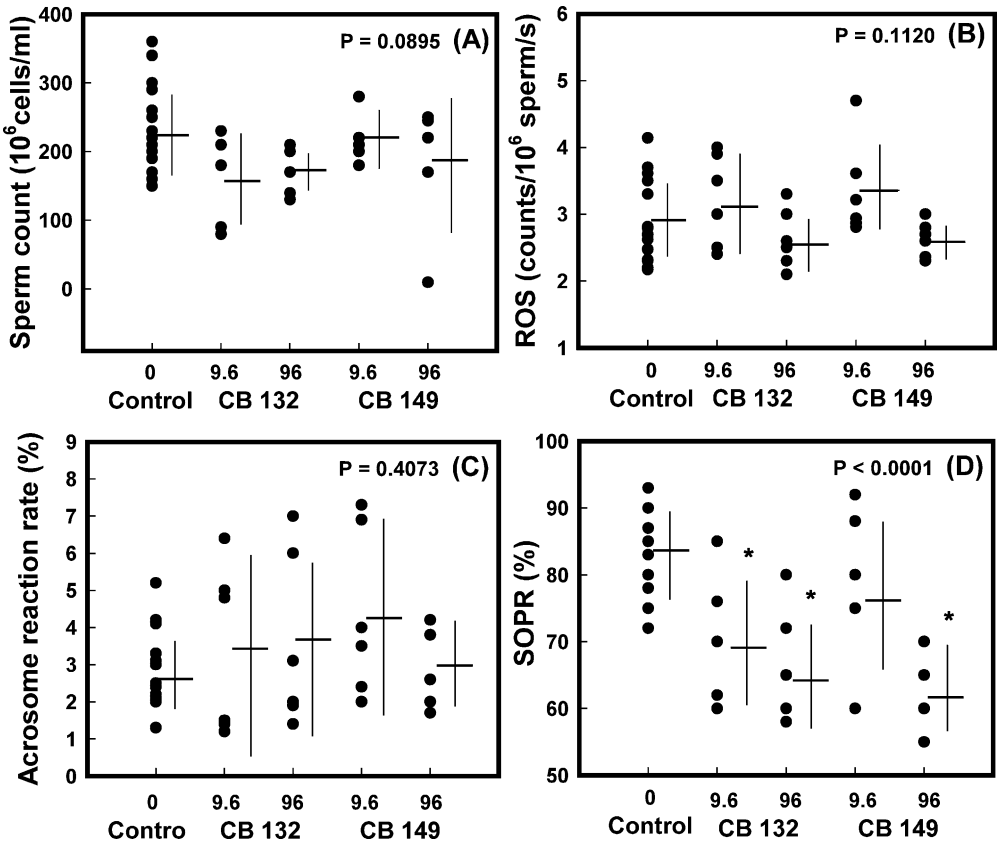

Fig. 2. Sperm counts (A), ROS generation (B), acrosome reaction rate (C), and SOPR (D) were analyzed and expressed as individual values in the control group ($n = 16$), 9.6 mg/kg of CB 132 ($n = 6$), 96 mg/kg of CB 132 ($n = 6$), 9.6 mg/kg of CB 149 ($n = 6$), and 96 mg/kg of CB 149 ($n = 6$) dose groups. Because of identical values, the number of points might be less than 16 (control group) or 6 (exposed groups) in some columns. Significant difference measured by the Tukey–Kramer honestly significantly difference (HSD) was * $P <$ 0.05 compared with the control group.

4. Discussion

By giving a single dose of 9.6 or 96 mg/kg of CB 132 and CB 149 at 21 days of age in male rats, serum levels of triiodothyronine as well as sperm motility, velocity and capability of penetrating oocytes were significantly decreased at 16 weeks of age. An estimate of the level of PCBs intake from breast milk was 0.23 and 2.3 mg/kg per day in the 9.6 and 96 mg/kg of CB 132 or CB 149 dose groups, respectively. The exposure level approaches the maximum FDA recommended level for PCB mixtures in food and breast milk (4 ppm, Maxim and Harrington, 1984). In term of evaluating the absorption dose of CB 132 and CB 149 exposure in environmental contamination, another study analyzed the CB congeners patterns in rats consuming diets containing Great Lakes salmon. After 19 weeks, concentration of CB 132 and CB 149 in adipose tissue was 0.06 and 0.22 mg/kg, and the adipose PCB patterns in the rats were similar to that observed in human populations (Jordan and Feeley, 1999).

Possible effects of postnatally exposed to non-dioxin-like CB congeners on sperm quality are less documented in animals and human studies. In a previous study, we reported hepatic enzyme induction and acute hormone effects of CB 132 and CB 149 in immature female rats received the same dose of 9.6 and 96 mg/kg treatment and at the same time of exposure (Li et al., 2001). The readily metabolized CB 132 did not cause any significant

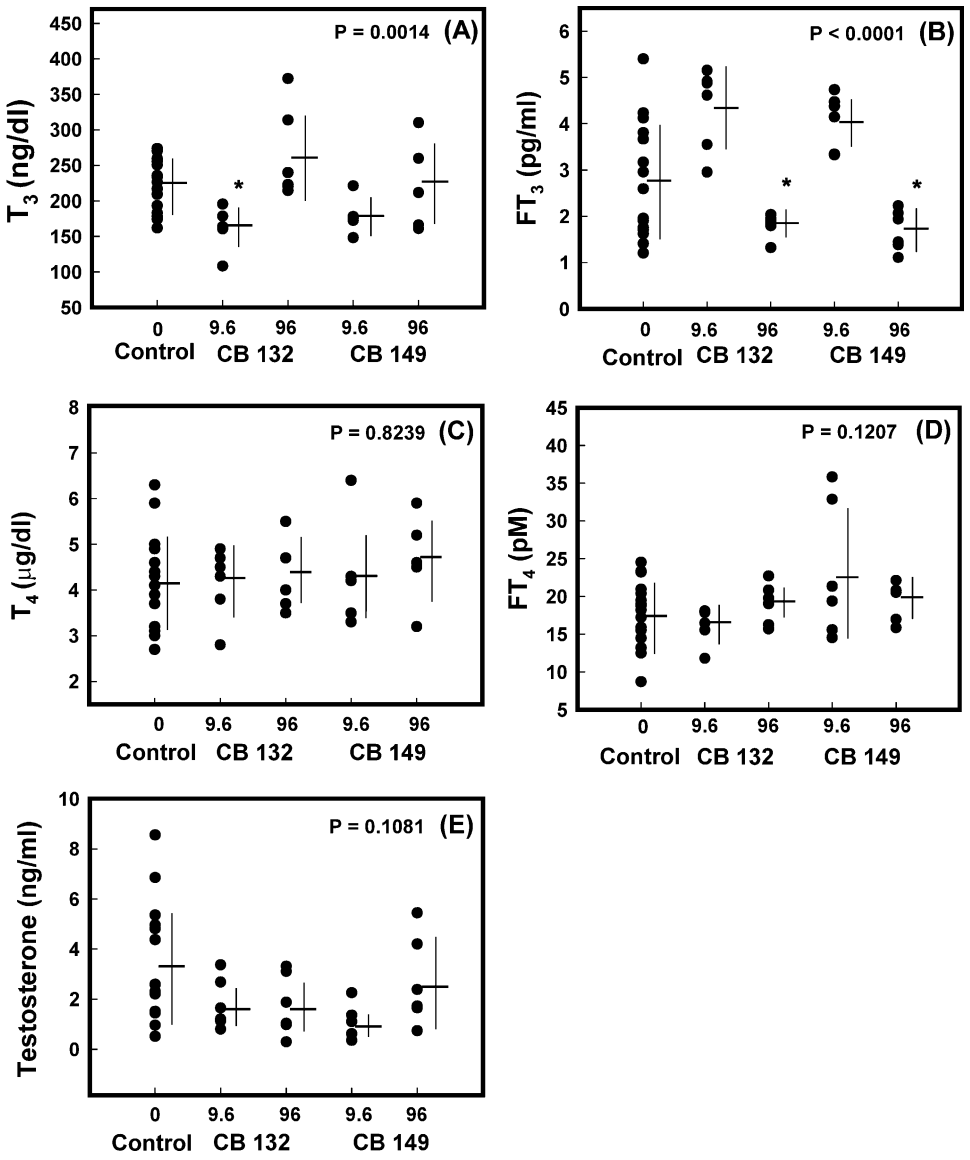

Fig. 3. Serum level of T_3 (A), FT_3 (B), T_4 (C), FT_4 (D), and TT (E) were measured and expressed as individual values in the control group ($n = 16$), 9.6 mg/kg of CB 132 ($n = 6$), 96 mg/kg of CB 132 ($n = 6$), 9.6 mg/kg of CB 149 ($n = 6$), and 96 mg/kg of CB 149 ($n = 6$) dose groups. Because of identical values, the number of points might be less than 16 (control group) or 6 (exposed groups) in some columns. Significant difference measured by the Tukey–Kramer honestly significantly difference (HSD) was * $P < 0.05$ compared with the control group.

increase in all endpoints measured in hepatic enzyme induction test. CB 149 was a weak 7-pentoxyresorufin O-dependentylase (PROD) and 7-benzyloxyresorufin O-debenzylase (BROD) inducer and a modest depleter of serum thyroxine in prepubertal female rats (Li et al., 2001). Findings of the effects of postnatal CB 132 and CB 149 exposure on male reproductive toxicity were different to the performance on hepatic toxicity.

We found that the sperm motility, VCL, VAP, VSL, ALH, BCF, and fertilizing ability were significantly affected and sperm count was slightly

changed in rats postnatalty exposed to CB 132 and CB 149. Of primary importance of the fertilizing sperm to work in sequence is progressive forward sperm motility. This particular attribute is dependent on proper mitochondrial function (Auger et al., 1989). A decrease of 13% in normal liver mitochondria volume was observed in rats exposed to 50 ppm of CB 153 in diet (Peng et al., 1997). Whether and how the CB 132 and CB 149 altered sperm mitochondrial functions is still unclear. Timing of exposure may influence the observed effects on spermatogenesis and maturation. However, information is lacking on comparisons between prenatal and postnatal exposure to non-dioxin like CB congener in male reproduction. The exposure time of this study is on days 23 after birth, between neonatal and pubertal period, called 'prepuberty'. In perpubertal stage, the spermatogenesis might be more susceptible to endocrine disruptors because early stages of development are occurring (Clermont and Perey, 1957) and the blood-testes barrier is not fully developed (Setchell and Brooks, 1988). Early postnatal exposure to PCBs on days 1, 3, 5, 7 and 9 of lactation affected ability of sperm to fertilize eggs but not production, morphology or motility of epididymal sperm (Sager et al., 1991). In 18-weeks-old rats following prenatal exposure to Aroclor 1254, adverse affects on fertility, decreased the ventral prostate and seminal vesicle weight, and enlarged testes were observed, but the sperm functions were not measured in that study (Sager, 1983). Another study found that epididymal sperm count and velocity were increased at 16 weeks of age after prenatal and lactational exposure to 25 mg/kg of Aroclor 1242. However, sperm fertilizing ability in vitro was significantly decreased in all Aroclor 1242-exposed group at 16 and 45 weeks of age (Fielden et al., 2001). Epidemiological study found that adverse effects on human reproduction have also been observed in young men exposed prenatally to PCBs/PCDFs, including sperm motility, velocity, BCF and ability to bind and penetrate the hamster oocyte (Guo et al., 2000). Genital development and pubic hair growth were inversely correlated with the serum concentration of CB 138, 153, and 189 in boys, but the CB 132 and CB 149 were not measured in that study (Den Hond et al., 2002). This study provided the direct evidence that male reproductive effects of CB 132 and CB 149 might contribute to the early pubertal exposure to non-dioxin-like CB congeners in which similar to the effects of dioxin-like CB congeners and causing observed impairments of sperm quality.

Plasma T_4 levels were significantly reduced in male rats following subchronic PCB exposure (Gray et al., 1993) but not in adult male offspring after gestational and/or lactational exposure to PCBs (Kim et al., 2001). Although reduced serum T_4 with normal T_3 has been reported in most PCBs exposure (Bastomsky et al., 1976; Ness et al., 1993), there were some studies which showed PCB effects on both T_3 and T_4. For example, serum T_3 and T_4 were depressed by chronic exposure of PCB mixtures (Byrene et al., 1987) as well as by acute exposure of coplanar PCBs (Desaulniers et al., 1997). The toxic effect of dioxin-like PCBs on thyroid is attributable to the structural similarities shared between PCBs and thyroid hormones (McKinney, 1989). In this study, T_4 and free T_4 levels were unaffected by both CB 132 and CB 149-treated groups. T_3 was significantly reduced in 9.6 mg/kg of CB 132 group. On the other hand, a significant decrease in free T_3 concentration was found both in 96 mg/kg of CB 132 and CB 149 groups compared with the controls, respectively. This is not a usual observation in non-dioxin-like PCB-treated rats. Whether this is caused by reduction in or competition for the serum binding protein for T_3 is unclear and will be a subject for future study.

In conclusion, our findings suggest that postnatally exposed to CB 132 and CB 149 might cause a decrease in sperm motility, VCL, VAP, VSL, ALH and BCF in 96 mg/kg dose groups. Serum T_3 level was decreased in 9.6 mg/kg of CB 132 group and free T_3 was decreased both in 96 mg/kg of CB 132 and CB 149 groups. In exposed group, data outside the control range may be obviously considered as pathological status. Although some of the exposed data points are clustered at the lower limit of the control range, this might indicate a beginning trend of an effect and which might be verified with even higher doses. The discrepancies in sperm function between CB 132 and CB 149-

treated rats could partially be caused by different half lives of these two compounds, resulting in different exposure levels in the critical periods of spermatogenesis in these two treatment groups. More studies concurrently on kinetics of these compounds are warranted. We now have learned that sperm motility, velocity, fertilizing capability, and serum T_3 in rats is far more susceptible to CB 132 and CB 149 when exposure occurs before pubertal stage. These finding might raise the concern that reproductive system in men may be more susceptible to non-dioxin-like CB congeners than previously believed. Whether findings in the SOPR can be interpreted as fertility changes warrant further investigation.

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