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Lactational exposure to dioxin-like polychlorinated biphenyl 169 and nondioxin-like polychlorinated biphenyl 155: effects on rat femur growth, biomechanics and mineral composition

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AhR - aryl hydrocarbon receptor

Cort – cortical bone

CortCSA – cortical bone cross-sectional area

CSA - cross-sectional area

ENDO – endosteal circumferences

ER - estrogen receptor

I_{min} - minimum value of moment of inertia

ICP-MS - inductively coupled plasma-mass spectrometry

PCB – polychlorinated biphenyls

PERI – periosteal circumference

PND – postnatal day

s-ALP – serum activity of alkaline phosphatase

s-ALT - serum activity of alanine aminotransferase

s-AST - serum activity of aspartate aminotransferase

s-Ca - serum calcium

s-LDH - serum activity of lactate dehydrogenase

s-PO₄ - serum inorganic phosphate

TCDD - 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

tCSA – total cross-sectional area

TEF - toxic equivalency factor

TEQ - toxic equivalent

TWI - tolerable weekly intake

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ABSTRACT

Exposure to polychlorinated biphenyls (PCBs), which are persistent lipophilic environmental pollutants, has a variety of adverse effects on wildlife and human health, including bone mineralization, growth and mechanical strength. The present study evaluated the effects of lactational exposure to nondioxin-like PCB-155 and dioxin-like PCB-169, individually and in combination, on pubertal rat femur development and its biomechanics. After offspring delivery, Wistar rat mothers were divided into four groups, i.e., PCB-169, PCB-155, PCB-155+169 and control, and were administered PCBs intraperitoneally. Data on bone geometry, biomechanics and mineral composition were obtained by analysis of femurs from 42-day-old offspring by microCT scanning, three-point bending test and inductively coupled plasma mass spectrometry. Decreased somatic mass and femur size, i.e., mass, periosteal circumference and cross sectional area, were observed in the PCB-169 and PCB-155 groups. Additionally, lactational exposure to planar PCB-169 resulted in harder and more brittle bones containing higher amounts of minerals. Combined exposure to structurally and functionally different PCBs demonstrated only mild alterations in bone width and mineralization. To conclude, our results demonstrated that alterations, observed on postnatal day 42, were primarily induced by PCB-169, while toxicity from both of the individual congeners may have been reduced in the combined group.

Key words: polychlorinated biphenyls; bone; biochemical parameters; geometry; biomechanics; rats

1. INTRODUCTION

Production of polychlorinated biphenyls (PCBs), which are persistent lipophilic environmental pollutants, has been banned for decades; however, they are still found worldwide including contaminated soil in India (Murugan and Vasudevan, 2017), woodchips boilers in Taiwan (Bai et al., 2017), polar bears in the Arctic Canada (Letcher et al., 2017), dolphins in Mediterranean sea (Jepson et al., 2016), cave salamander (*Proteus anguinus anguinus*) in the Slovenian karstic hinterland (Pezdiric et al., 2011), professionally exposed workers from a transformer recycling company (Schettgen et al., 2012) and former underground miners in Germany (Schettgen et al., 2018). Despite a general decrease in dietary exposure of dioxins and PCBs confirmed by the 2012 comprehensive monitoring data of 26 European countries, it is estimated that between 1.0% and 52.9% of individuals are still exposed to levels above the tolerable weekly intake (TWI) of 14 pg toxic equivalent (TEQ)/kg body weight, with toddlers/children being the most exposed groups (Malisch and Kotz, 2014). Due to continued biomagnification of PCBs along the food chain, food consumption remains the major source of PCB exposure, representing 90%–98% of the average exposure. However, it should be emphasized that during 6 months of breast-feeding, an infant receives an estimated 6.8–12% of its lifetime PCB body burden and therefore, TWI levels are not applicable to breastfed infants/suckling animals (ATSDR, 2000; Patandin et al., 1999).

The Slovenian Human Biomonitoring Surveillance Programme showed that between 2011 and 2014, there were no exceeded levels of indicator PCBs in human milk and serum, i.e., PCB 28, 52, 101, 118, 138, 153 and 180, with the highest levels being found in regions where industrial pollution was heavily present 30 years ago

(Horvat et al., 2015; Jan and Tratnik, 1988; Perharic and Vracko, 2012). A recent German study also revealed a reduction in PCB body burden compared to earlier studies (Schettgen et al., 2015). In China, a long-term (1930-2100) dynamic simulation of human exposure to PCBs predicted an approximate 30-year delay of nondioxin-like PCB-153 peak body burden in an adult Chinese woman compared to a European counterpart (Zhao et al., 2018).

Most PCB studies have investigated the effects of their commercial mixtures, e.g., Aroclors 1254 and 1221 (Kutlu et al., 2007; Yilmaz et al., 2006), Clophen (Pereira and Rao, 2007), Pyralene (Vrecl et al., 1995), and dioxin-like PCB-126 (Alvarez-Lloret et al., 2009; Lind et al., 1999). In particular, PCB-126, the most toxic PCB congener with a toxic equivalency factor (TEF) of 0.1 (van den Berg et al., 2006), demonstrated (anti-estrogenic) effects similar to those of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most potent known aryl hydrocarbon receptor (AhR) agonist (Safe, 1994; Van der Burght et al., 2000). Due to the chemical similarity of nondioxin-like PCBs to estradiol 17- β , studies suggested their induction of estrogenic effects through their binding to estrogen receptor (ER) (Cooke et al., 2001; Dempster, 2008; Geyer et al., 2000). Combined exposure to either TCDD and PCBs or individual PCB congeners resulted in altered pharmacokinetic behavior (Lee et al., 2007, 2002) and additive, synergistic, or antagonistic effects depending on the dose and selection of toxicity end points (Lu et al., 2009; van Birgelen et al., 1996). Exposure to PCBs and environmental organochlorines has been shown to affect bone mineral density in humans (Hodgson et al., 2008) and wild animals including polar bears (Daugaard-Petersen; Sonne et al., 2015), Baltic grey seal (Lind et al., 2003) and freshwater turtle (Ming-Ch'eng Adams et al., 2016). Bone studies in experimental animals reported biochemical and toxicological responses after exposure to TCDD (Herlin et al., 2013; Jämsä et al., 2001;

Lind et al., 2009; Nishimura et al., 2009), environmental and commercial mixtures of chemical pollutants (Daugaard-Petersen et al., 2018; Elabbas et al., 2011a, 2011b; Ramajayam et al., 2007) or individual PCBs (Brankovič et al., 2017; Gutleb et al., 2010; Lind et al., 2000). In young animals, after *in utero* and lactational exposure to TCDD, bone growth and mechanical strength were affected in wild-type AhR rats on postnatal day (PND) 40 (Miettinen et al., 2005), and bone mineralization was impaired in mice on PND 21 (Nishimura et al., 2009). However, these alterations were more prominent with combined *in utero* and lactational exposure than with lactational exposure alone (Miettinen et al., 2005). The above-mentioned effects have mostly been reported for long bones (femur, tibia, and humerus). Additionally, exposure to TCDD and dioxin-like PCB-169 also affected bones that ossify with intramembranous ossification. Few studies have reported the effects on skull craniofacial and mandibular growth, i.e., reductions in skull size, facial length and vault breadth, in adult rats and rat offspring on PND 9 and 22 (Grošelj et al., 2014; Sholts et al., 2015). Studied PCB-155 (2,2',4,4',6,6') and PCB-169 (3,3',4,4',5,5') have different planarity and toxicity. Compared to PCB-155, the more lipophilic dioxin-like PCB-169 (TEF 0.03 (van den Berg et al., 2006)) showed higher excretion in ovine milk (Vrecl et al., 2005) and higher residual levels in the mandibular bone of lactationally exposed lambs (Jan et al., 2013).

Despite a decade of experiments on PCB effects in bone tissue, information on its biomechanical behavior is still not comprehensive and often opposing. Our preceding study on prepubertal rat offspring (PND 22) revealed inhibitory effects of dioxin-like PCB-169, but not nondioxin-like PCB-155, on femur growth and mineralization (Brankovič et al., 2017). However, we encountered problems when performing biomechanical measurements on the developing prepubertal bones, i.e., on PND 22, femurs were insufficiently mineralized to be precisely assessed in a three-

132 point-bending test (Brankovič et al., 2017). This challenge was not the case in rats from
133 PND 35 onwards (Finnilä et al., 2010; Indrekvam et al., 1991). Therefore, the aim of
134 the current study was to evaluate the effects of PCB-169 and -155, exposed
135 lactationally, on rat femur growth, density and biomechanical behavior of older (early
136 pubertal) 6-week-old animals.

2. MATERIALS AND METHODS

2.1 Animals and treatment

The animals were maintained in accordance with the Slovenian Animal Protection Act (Official Gazette of the Republic of Slovenia, No. 43/2007) and the Directive 2010/63/EU. Animal handling procedures were approved by the ethical committee of the Veterinary Administration of the Republic of Slovenia (Permit No. 3440-165/2006). Briefly, mature female Wistar rats (age 9 ± 1 week, body mass (b.m.) 240 ± 10 g) obtained from Lek d.d., Slovenia were individually housed and maintained on a 12-h light/dark cycle at a constant room temperature and humidity after mating. A cereal-based fixed-formula maintenance diet for the rats (Altromin 1324, Lage, Germany) and water were administered *ad libitum*. After delivery, the mothers and their offspring were randomly divided into four groups and the PCB congener administration regimes with loading and maintenance doses were executed as previously described (Brankovič et al., 2017; Grošelj et al., 2014). This protocol was based on the TEF of PCB-169 (van den Berg et al., 2006) and the previously established excretion patterns of PCB-155 and PCB-169 in milk (Vrecl et al., 2005). Briefly, PCB-155 and PCB-169 (Promochem, Wesel, Germany), dissolved in olive oil, were administered intraperitoneally to lactating rats, with the first dose (the loading dose) given to mothers on the day of delivery (day 0), followed by subsequent maintenance doses. The PCB-155 group was administered a loading dose of PCB-155 (6 mg/kg b.w.) followed by three maintenance doses (2 mg/kg b.w.) on days 6, 12, and 17 after delivery; the total amount of PCB-155 administered was 12 mg/kg b.w. The PCB-169 group was administered a loading dose

of PCB-169 (2 mg/kg b.w.) followed by two maintenance doses (0.5 mg/kg b.w.) on days 6 and 14 after delivery; the total amount of PCB-169 given was 3 mg/kg b.w. (*i.e.*, 90 µg TEQ/kg b.w.). The combined regime of PCB-155 and PCB-169 administration was used in the PCB-155+169 group, *i.e.*, a loading dose of 2 mg of PCB-169/kg b.w. and 6 mg of PCB-155/kg b.w. followed by the abovementioned maintenance doses of PCB-155 and PCB-169. The control group was administered 0.5 mL of olive oil (vehicle) on the day of delivery (day 0) followed by four applications of 0.15 mL of olive oil on days 6, 12, 14 and 17. On PND 22, offspring were separated from their mothers, corresponding to the average weaning age for rats (3 weeks); however, the consumption of pelleted (solid) food (Altromin 1324, Lage, Germany) started before, as time spent suckling begins to decline around PND 20 (Baker et al., 1979). On PND 42, offspring were weighted to the nearest 0.01 g (scale ET-1111, Slovenia), checked for gender and euthanized. Blood was collected from the ophthalmic plexus and left femurs were dissected and frozen at -80 °C until measurements.

2.2 Femur Geometry

Thawed femurs were evaluated using a microCT scanner (Siemens Inveon MultiModality, Knoxville, TN, USA) and “Inveon Research Workplace” software (Siemens Medical Solutions USA, Inc., PA, USA). The procedure and protocols were conducted as reported previously (Brankovič et al., 2017). Femur length was measured from the top of the femur head to the most distal point of the medial condyle. For measuring cortical bone parameters at mid-diaphysis, a tomographic slice of 18.9 µm effective voxel size perpendicular to the femur's long axis was chosen at the site distanced 45% from the top of the femur head. Cortical bone thickness (in mm) was

measured using RadiAnt DICOM Viewer© (Medixant, Poznan, Poland). Cross-sectional area (CSA) of total bone (tCSA), cortical bone (CortCSA) and the medullary area (in mm²), together with periosteal and endosteal circumferences (PERI and ENDO, mm), were assessed using ImageJ 1.43. The ratio of cortical to total bone thickness and of femur mass to length were calculated. Cortical bone density (mg/cm³) at the left diaphysis femur was measured using a microCT hydroxyapatite phantom (MicroCT-HA D32, serial No. MHA-102, QRM, Moehrendorf, Germany) containing five inserts of different hydroxyapatite densities. For biomechanical calculations, the inner and outer radius of long and short cross-sectional axis were measured (in mm) at the point where the press head load was applied (at 50% femur length).

2.3 Biomechanical properties

Biomechanical properties of left femurs were determined by a three-point bending test on a universal testing machine (4301, Instron Corp., Canton, MA, USA) as described previously (Brankovič et al., 2017). Each femur was loaded perpendicularly to its longitudinal axis at the mid-diaphysis on a custom-made press head (diameter 2 mm). Femurs were positioned at two holders (diameter 3 mm) with span length of 12 mm, with the cranial surface facing upwards. The load was applied at a constant rate of 0.5 mm/min. The load (N) and displacement (mm) data were recorded at a sampling rate of 10 Hz until fracture. Stiffness (N/mm) was calculated as the initial linear slope and energy absorption (Nmm) as the area under the load-displacement curve. Ultimate stress to fracture (flexural strength, in MPa) and Young's modulus (in MPa) were calculated as described previously (Brankovič et al., 2017). To evaluate flexural strength and Young's modulus of the bone material, the corresponding minimum value

of the moment of inertia ($I_{\min}$, in mm^4) for the most loaded cross-section was calculated (Jepsen et al., 2015).

2.4 Blood and Bone Analyses

Biochemical parameters in serum, i.e., calcium (s-Ca, mmol/L), inorganic phosphate levels (s- PO_4 , mmol/L) and the alkaline phosphatase (s-ALP), aspartate aminotransferase (s-AST), alanine aminotransferase (s-ALT) and lactate dehydrogenase activity (s-LDH, $\mu\text{kat/L}$ for all the enzymes) were determined using an automatic chemistry analyzer at the University Medical Center Ljubljana (Olympus Corp., Hamburg, Germany). PCB congeners levels in blood were determined by the solid phase micro extraction technique and gas chromatography with electron capture detection as described previously (Vrecl et al., 2005). After the three-point bending test, femurs were weighed (Mettler Toledo, AX205DR/A, Uznach, Switzerland) and placed in a muffle furnace (Nabertherm, Bremen, Germany) to be burned at 550°C overnight. The ash mass of the femur (mg) was determined, and calcium (Ca), phosphorus (P) and magnesium (Mg) contents (%) were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) using an ICP-mass spectrometer (Varian 820-MS, Mulgrave, Australia) similar to our previous report (Brankovič et al., 2017). Inorganic content (ash mass to femur mass, in %) and Ca/P ratio were calculated.

2.5 Statistical analysis

Data were analyzed using SPSS 20 for Windows (SPSS Inc., Chicago, Ill, USA). Tables displayed median with IQR. We predicted an interaction between the PCBs and the animals' body mass. Due to 5-7 animals/experimental group, differences between groups were analyzed by nonparametric Kruskal-Wallis test (p -values less than 0.05 were considered to be statistically significant), followed by Wilcoxon rank sum test as *post hoc* test for comparing three PCB-exposed groups to the control. When using Wilcoxon *post hoc* test, a Bonferroni correction was applied (p -value less than 0.0167 was used as our critical level of significance).

3. RESULTS

3.1 PCB congener levels in the offspring

The presence of PCB-155 and PCB-169 two weeks after the end of weaning was confirmed in the offsprings' blood on PND 42. The mean PCB congener levels were 0.50 ng/mL of PCB-169 and 2.20 ng/mL of PCB-155 in the individual PCB groups and 0.25 ng/mL of PCB-169 and 1.70 ng/mL of PCB-155 in the combined PCB-155+169 group. Studied congeners were not detected in the control group.

3.2 Femur length and cortical properties at mid-diaphysis

Total body mass, femur length, and morphometric parameters at mid-diaphysis are summarized in Table 1. When we analyzed females and males separately similar differences between experimental groups appeared in both sexes (data not shown). Compared to the control group, body mass of the animals was decreased in the PCB-169 ($p < 0.001$) and PCB-155 groups ($p = 0.010$) and the total femur length was decreased in the PCB-169 group ($p < 0.001$). When we adjusted femur mass to its length, the decrease was significant in the PCB-169 ($p = 0.003$) and PCB-155 groups ($p = 0.007$).

At mid-diaphysis, periosteal circumference was decreased in the PCB-169 ($p = 0.001$) and PCB-155 groups ($p = 0.011$) and endosteal circumference in the PCB-169 group ($p = 0.002$). All three CSA parameters were lowered in the PCB-169 group ($p =$

0.001) and total CSA in the PCB-155 group ($p = 0.004$). Cortical bone thickness was decreased in the PCB-169 ($p = 0.001$), PCB-155 ($p = 0.002$) and PCB-155+169 groups ($p = 0.001$). The thickness ratio between cortical and total bone was lowered in the PCB-155+169 group ($p = 0.003$). Cortical bone density was increased in the PCB-155 ($p = 0.004$) and PCB-155+169 groups ($p = 0.005$).

Table 1. Somatic body mass, femur geometric and morphometric properties (median with IQR) of Wistar rat offspring on PND 42 in the different exposure groups. Number of femurs used is given in parentheses.

Parameter	CONTROL (7)	PCB-169 (7)	PCB-155 (7)	PCB-155+169 (5)	p-value
Body mass (g)	142.7 (27.4)	107.2 (14.6) ^a	96.4 (45) ^a	127.8 (32.7)	0.001
Femur length (mm)	24.7 (0.8)	21.9 (1.1) ^a	23.4 (1.2)	24.0 (0.8)	< 0.001
Femur mass/length (mg/mm)	10.95 (0.81)	9.12 (1.35) ^a	9.79 (1.04) ^a	11.73 (1.26)	< 0.001
PERI (mm)	9.09 (0.42)	8.03 (0.34) ^a	8.66 (0.52) ^a	8.88 (0.58)	< 0.001
ENDO (mm)	6.87 (0.82)	5.88 (0.33) ^a	6.42 (0.37)	6.38 (0.64)	0.001
tCSA (mm ²)	6.03 (0.66)	4.79 (0.39) ^a	5.35 (0.70) ^a	5.77 (0.80)	< 0.001
CortCSA (mm ²)	2.64 (0.29)	2.21 (0.15) ^a	2.35 (0.46)	2.47 (0.34)	< 0.001
Medullary area (mm ²)	3.45 (0.84)	2.55 (0.27) ^a	3.04 (0.37)	3.02 (0.67)	< 0.001
Cort thickness (μm)	310 (10)	250 (30) ^a	280 (20) ^a	270 (10) ^a	< 0.001
Cort/Total thickness (%)	23.7 (2.4)	21.7 (3.7)	23.0 (0.9)	21.1 (1.9)	0.006
Cort density (mg/cm ³)	1859 (36)	1882 (82)	1924 (38) ^a	1995 (55) ^a	0.001

The statistical significance of differences between groups was analyzed by Kruskal-Wallis test, followed by Wilcoxon rank sum test. PERI – periosteal circumference, ENDO – endosteal circumference, CSA - cross-sectional area, tCSA – total CSA, CortCSA – cortical bone CSA, Cort – cortical bone. ^a – significantly different from the control group ($p \leq 0.0167$)

3.3 Femur Biomechanical Properties

Biomechanical properties analyzed from the load-displacement curves shown in Fig. 2, as obtained by the three-point bending test of each bone specimen, are summarized in Table 2. Compared to the control group, no statistically significant difference was observed in the load to fracture ($p = 0.676$) and stiffness ($p = 0.085$) between the experimental groups. In the PCB-169 group, flexural strength was increased ($p = 0.001$), I_{min} was lowered ($p = 0.001$) and Young's modulus showed an increasing trend, but did not reach statistical significance ($p = 0.017$). Displacement was increased in the PCB-155+169 group ($p = 0.016$).

Fig. 1. Load-displacement curves obtained by the three-point bending test for Wistar rat offspring femurs on PND 42 in the different exposure groups. Number of femurs used is given in parentheses.

Table 2. Results of the three-point bending test of the femur mid-diaphysis (median with IQR) of Wistar rat offspring on PND 42 in the different exposure groups. Number of femurs used is given in parentheses.

Parameter (n)	Control (7)	PCB-169 (7)	PCB-155 (7)	PCB-155+169 (5)	p-value
Load to fracture (N)	52.1 (8.7)	51.6 (12.0)	48.2 (13.3)	57.4 (24.1)	ns
Displacement (mm)	0.29 (0.10)	0.30 (0.09)	0.23 (0.05)	0.37 (0.07) ^a	0.003
Flexural strength (MPa)	90.2 (27.0)	161.3 (39.2) ^a	105.5 (37.3)	117.1 (51.5)	0.003
Young's modulus (MPa)	3673 (1837)	5550 (2237)	4730 (1258)	3835 (1727)	0.037
$I_{\min}$ (mm ⁴)	2.15 (0.53)	1.35 (0.44) ^a	1.84 (0.46)	1.92 (0.57)	< 0.001
Stiffness (N/mm)	228 (84)	201 (66)	248 (69)	212 (74)	ns
Energy absorption (Nmm)	7.4 (3.8)	10.5 (5.3)	7.2 (1.7)	12.4 (5.6)	0.006

The statistical significance of differences between groups was analyzed by Kruskal-Wallis test, followed by Wilcoxon rank sum test. $I_{\min}$ - cross-sectional moment of inertia (the minimum value).
^a – significantly different from the control group ($p \leq 0.0167$), ns – not significant

3.4 Serum Biochemical Analysis and Liver Enzymes

Compared to the control group (Table 3), no statistically significant difference was observed in s-ALT level ($p = 0.194$). In the PCB-169 group, levels of s-AST ($p < 0.001$) and s-ALP ($p = 0.001$) were increased, and in the PCB-155 group, level of s-AST was increased ($p < 0.001$). S-Ca was increased in the PCB-169 group ($p = 0.002$) and decreased in the PCB-155 ($p = 0.002$) and PCB-155+169 groups ($p = 0.016$).

Table 3. Serum concentration of biochemical parameters level/activity: calcium (s-Ca), phosphate (s-PO₄), total nonspecific alkaline phosphatase (s-ALP), aspartate aminotransferase (s-AST) and alanine aminotransferase (s-ALT) (median with IQR) of Wistar rat offspring on PND 42 in the different exposure groups. Number of femurs used is given in parentheses.

Parameter (n)	Control (7)	PCB-169 (7)	PCB-155 (7)	PCB-155+169 (5)	p-value
s-Ca (mmol/L)	2.66 (0.12)	2.77 (0.12) ^a	2.46 (0.14) ^a	2.54 (0.18) ^a	< 0.001
s-PO ₄ (mmol/L)	3.70 (0.36)	3.82 (0.49)	3.26 (0.78)	3.35 (0.36)	0.008
s-AST (μkat/L)	1.87 (0.51)	2.27 (0.36) ^a	2.46 (0.42) ^a	2.38 (1.58)	0.001
s-ALT (μkat/L)	0.97 (0.36)	1.17 (0.32)	1.16 (0.29)	1.21 (0.67)	ns
s-ALP (μkat/L)	4.28 (1.11)	6.28 (2.46) ^a	3.38 (1.52)	4.72 (2.31)	< 0.001

The statistical significance of differences between groups was analyzed by Kruskal-Wallis test, followed by Wilcoxon rank sum test.

^a – significantly different from the control group ($p \leq 0.0167$), ns – not significant

3.5 Femoral Ash Mass and Mineral Composition

Bone analyses are summarized in Table 4. Compared to the control group, ash mass was decreased in the PCB-169 ($p < 0.001$) and PCB-155 groups ($p = 0.005$). Calculated inorganic content in the bone (ash/total femur mass) was decreased in the PCB-155 ($p = 0.006$) and PCB-155+169 groups ($p = 0.005$). The increased levels of elements in ash (Ca, P and Mg) were detected in the PCB-169 group ($p < 0.001$).

Table 4. Biochemical analysis of the parameters of inorganic femur content (median with IQR) of Wistar rat offspring on PND 42 in the different exposure groups. Number of femurs used is given in parentheses.

Parameter (n)	Control (7)	PCB-169 (7)	PCB-155 (7)	PCB-155+169 (5)	p-value
Ash mass (mg)	125 (15)	93 (16) ^a	102 (21) ^a	122 (12)	< 0.001
Inorganic content (%)	47.1 (3.0)	46.2 (2.1)	44.9 (1.1) ^a	43.3 (3.3) ^a	0.001
Ca/P ratio ¹	1.83 (0.03)	1.88 (0.07)	1.88 (0.03) ^a	1.86 (0.02)	0.039
Ca in ash (%) ¹	30.2 (0.9)	34.2 (0.9) ^a	32.2 (3.3)	31.0 (0.5)	< 0.001
P in ash (%) ¹	16.5 (0.3)	18.2 (0.8) ^a	17.0 (1.3)	16.7 (0.2)	< 0.001
Mg in ash (%)	0.75 (0.04)	0.85 (0.09) ^a	0.80 (0.07)	0.78 (0.02)	< 0.001

The statistical significance of differences between groups was analyzed by Kruskal-Wallis test, followed by Wilcoxon rank sum test.

^a – significantly different from the control group ($p \leq 0.0167$)

4. DISCUSSION

Our objective was to examine the individual and combined effects of nondioxin-like PCB-155 and dioxin-like PCB-169 on femur growth, mineralization and biomechanics in 42-day-old rat offspring. Analysis indicated decreased somatic mass and femur size, i.e., mass, periosteal circumference and cross sectional area, in animals lactationally exposed individually to either PCB-169 or PCB-155 compared to the control; however, the most pronounced effects, including decreased femur length, were detected in the PCB-169 group. Additionally, only in the PCB-169 group compared to the control, cortical bone tissue appeared to be more brittle and harder, containing higher amount of minerals. In the combined PCB-155+169 group, the majority of studied parameters were largely comparable with those obtained for the control.

We previously reported decreases in body mass and total femur length adjusted for body mass in the PCB-169 and PCB-155+169 groups on PND 22 (Brankovič et al., 2017). By PND 42, the body mass also significantly decreased in the PCB-155 group but not in the combined group (Table 1). This finding in the combined group can be attributable to high body mass data dispersal in this group, although similar to our observation, nondioxin-like PCB-153 antagonized TCDD-induced body mass loss in mice (Bannister and Safe, 1987). A constant reduction in somatic growth from weaning until adulthood was reported after exposure to a mixture of dioxin- and nondioxin-like PCB congeners -138, -153, -180 and -126 (Cocchi et al., 2009). Assessment of femur geometry showed ~~that in the PCB-169 group,~~ hindered appositional bone growth in the PCB-169 group that affected cortical bone and bone marrow area. Delayed bone growth in width in the PCB-155 group could only be detected at the periosteal surface,

which leads to narrower cortical bone thickness but not marrow area (Table 1). Since bone growth was more hindered on PND 42 compared to PND 22 (Brankovič et al., 2017), it could be assumed that the effects of PCB-155 and PCB-169 on appositional bone growth became detectable later than the effects on longitudinal bone growth. Hindered femur growth could be a result of delayed sexual maturation of the offspring on PND 42, since dioxin-like PCBs are known to be endocrine disruptors (Knutsen et al., 2018). However, sexual maturation of male rats was normal, i.e., on PND 42 spermatogenesis was fully developed with complete stages in seminiferous epithelium (Štrbenc et al., 2007) and no other signs of delayed puberty onset due to PCB-169 or PCB-155 exposure or their combination were observed. Therefore, it could be assumed that PCB affected somatic and femur growth directly and not through hindered sexual maturation.

Studies evaluating effects of PCBs on bones of pubertal animal are scarce. Similar to our findings, shorter and narrower femurs (decreased cortical or total CSA and circumferences) were reported after TCDD exposure *in utero* or lactationally on PND 35 (Finnilä et al., 2010; Miettinen et al., 2005) and on PND 40 (Miettinen et al., 2005). The same bone geometric parameters were decreased after *in utero* and lactational exposure to Aroclor 1254 (Elabbas et al., 2011b) or in perinatal exposure to environmental contaminants, including 14 PCB congeners (Elabbas et al., 2011a). Interestingly, in these studies, the above-mentioned effects on bones were reversible and disappeared a certain time after exposure (after the elimination of compounds from the body by PND 77 and PND 350) (Elabbas et al., 2011a, 2011b; Finnilä et al., 2010; Miettinen et al., 2005). Additionally, no tendency of accelerated femur growth possibly induced by the nondioxin-like PCB-155 on PND 22 (Brankovič et al., 2017) was detected later on PND 42. Lundberg et al. (2006) and Gutleb et al. (2010) studied

effects of nondioxin-like PCB-118 and PCB-153 on offspring bones and reported shorter long bones in goat offspring and ewe fetuses. Gender-dependent differences on PND 42 had not been present which could be explained by a study of the distal epiphyseal growth plate of the radius between PND 20 and 40 in rats (Hansson et al., 1972) and the reportedly unremarkable effects of estrogens and ER α on growth plate thickness in early puberty, contrary to the late phase of puberty (Borjesson et al., 2013).

The relative amount of bone inorganic substance was twofold higher on PND 42 compared to PND 22 (Brankovič et al., 2017) and suggested that by PND 42, femurs were almost completely mineralized (47% of inorganic content compared to 49% - reference values for adult animals (Morgulis, 1931)). When comparing ash bone composition of prepubertal rats on PND 22 (Brankovič et al., 2017) and pubertal rats from this study (Table 4), a substantial increase in Ca and P content occurred only in the PCB-169 group (26.2% Ca and 16.7% P on PND 22 compared to 34.2% Ca and 18.2% P on PND 42). Moreover, on PND 22, Ca level was decreased in the PCB-169 group, suggesting delayed mineralization, while on PND 42 there was a significant increase in Ca. PCB-169 effects could be attributed to affected bone metabolism/accelerated bone mineral storage that could reflect i) higher osteoid mineralization, ii) increased activity of osteoblasts or alternatively iii) hindered bone remodeling (suppressed effects on osteoclasts). Serum mineral levels also reflected bone mineral levels. Higher serum Ca level was detected in the PCB-169 group and lower levels in the PCB-155 and PCB-155+169 groups (Table 3). As previously reported, a slight increase in serum Ca levels was related to increased bone resorption (Andrews, 1989; Yilmaz et al., 2006), which was not observed in this study. Lind et al. (2000) reported shorter humeri with changes in composition of the organic matrix of rats exposed to PCB-126. Similar to PCB-169 in our study, PCB-126 did not affect

bone mineral density, but increased the organic and inorganic content of long bones. The study also demonstrated decreased water content and collagen concentration and increased concentration of pyridinoline cross-links between collagen molecules, important for their mechanical stability (Lind et al., 2000). Another study in rats exposed to PCB-126 reported a lower degree of mineralization, with increased trabecular, but not cortical, bone mineral density and alterations in the size and crystallinity properties of apatite crystals, which were explained by decreased serum levels of thyroid hormones and vitamin D found in the PCB-126 group (Alvarez-Lloret et al., 2009). Recent reviews and reports suggest a pleiotropic role of vitamin D in bone resorption and mineralization with negative or positive bone mineral regulation, depending on the physiological and pathological circumstances (Anderson, 2017) and species differences (e.g., stimulates human osteoblasts but inhibit murine ones) (van Driel and van Leeuwen, 2017). Increased s-ALP activity in the PCB-169 group (Table 3) might support the latter; however, we could not completely exclude possible hepatocellular injury due to increased s-AST activity (Jämsä et al., 2001, Kutlu et al., 2007, Yilmaz et al., 2006).

The three-point bending test revealed differences in the biomechanical performance among tested femurs through the respective load-displacement graphs (Fig. 1). Higher flexural strength, reached almost in the linear elastic range of deformation in bones exposed to PCB-169, suggested increased bone brittleness of this group (Table 2). Additionally, resistance to elastic deformation (high Young's modulus) in the PCB-169 group displayed stiffer cortical bone tissue. In contrast, parameters describing bone properties at the organ level, i.e., stiffness and energy absorption, revealed slight trends of increased energy absorption and decreased stiffness for the PCB-169 group. Since the whole-bone parameters depend on bone

size, the described trends could be associated with the smaller bones in the PCB-169 group (Table 1). The high energy absorption in the PCB-155+169 group is associated with the incidence of load-displacement curves exhibiting multiple (2) peaks and prolonged/high displacement (Fig. 1). This fracture behavior indicates the coexistence of several cracks that propagated through the cortex and coalesced to an ultimate fracture (Jepsen et al., 2015). In contrast to offspring on PND 22 (Brankovič et al., 2017), femur biomechanics on PND 42 reflected adverse effects of PCB-169 on bone, especially on the material itself (cortical bone tissue). Our previous study reported that femur biomechanical measurement accuracy using a three-point bending test on premature 22-day-old rats could not be adequately assessed due to insufficient mineralization and too thin bones (cortical/total bone thickness ratio) (Brankovič et al., 2017), while on PND 42 the test was adequately executed and the obtained results coincided with the geometry and mass spectrometry data. The capacity of bone to resist mechanical loading and fracturing depends on the quantity and quality of bone tissue. In bone extracellular matrix, the mineral phase provides the stiffness, while the collagen fibers are responsible for material flexibility – thereby providing the ductility and ability of the bone to absorb energy (Viguet-Carrin et al., 2006). Therefore, alterations of collagen properties (Lind et al., 2000) or mineral content (Alvarez-Lloret et al., 2009) can affect the mechanical properties of the bone. The reason for such material behavior could be explained by increased levels of Ca, P and Mg in femur ash of the PCB-169 group (Table 4), as mineral constituencies are responsible for the rigidity. Similarly, increased brittleness, ultimate strength and elastic modulus of mice femurs were associated with increased mineral-to-collagen ratio and reduced collagen content (Bi et al., 2016). Moreover, bone stiffness was decreased in the PCB-169 group (Table 2) due to the reduced bone cross sections (Table 1), and thus lowered

the moment of inertia (Table 2). Similar (lowered bending strength) was reported in developing (PND 35) and adult (PND 70) tibias after TCDD exposure (Finnilä et al., 2010). Even though we detected elevated cortical bone density in the PCB-155 and PCB-155+169 groups (Table 1), neither biochemical analysis (Tables 3 and 4) nor biomechanical parameters (Table 2) support these observations.

PCB-155 effects were detectable only in slower femur appositional growth, lighter (Table 1) and slightly less mineralized bone (Table 4), which did not lead to changed femur biomechanical properties (Table 2). The lack of alteration in most of the bone parameters in the PCB-155+169 group (Table 1-4), could indicate that toxicity from both of the individual congeners may have been reduced in the combined group, although the underlying mechanism is unclear. One of the possible explanations could be the approximately twofold lower serum PCB-169 level when coadministered with PCB-155 that resulted in milder outcomes on PND 22 (Brankovič et al., 2017) and the disappearance of the majority of PCB-169-induced effects by PND 42. In addition, PCB levels in all exposed groups were 5-fold lower compared to levels reported on PND 22 (Grošelj et al., 2014). Altered pharmacokinetic behavior was previously also reported after combinational exposure of either TCDD or planar together with nonplanar PCBs (Lee et al., 2007, 2002). Using the same PCB administration regime, in comparing suckling rat offspring on PND 22 (Brankovič et al., 2017) to pubertal rats on PND 42, we suggest that PCB effects on bone tissue geometry, composition and biomechanics are age-dependent.

When comparing the effect of PCB congeners/TCDD on bone growth and biomechanics the genetic background should also be considered as different rat strains could exhibit strain-specific responses (Sholts et al., 2014). In the past, experimental studies in animals were commonly performed with commercial PCB

mixtures/individual PCB congeners; whereas combined exposure to mixtures of environmentally relevant PCB congeners would more adequately reflect the exposure situation in humans and therefore warrants further studies.

Conclusions

Our data demonstrated that lactational exposure to dioxin-like PCB-169 was evident in puberty by decreased femur growth, and increased serum and bone mineral content resulting in stiffer and more brittle cortical bone. On PND 42, an inhibitory effect of less lipophilic nondioxin-like PCB-155 on bone growth was first observed. Animals of the combined group displayed a phenotype that was comparable to the control group in most of the studied parameters, suggesting there may be reduced toxicity from both of the individual congeners in the combined group.

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Conflict of Interests Statement

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