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Effects of *in utero* and lactational exposure to bisphenol A on bone tissue in rats

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SUMMARY

Bisphenol A (BPA) is an industrial chemical that is used in the production of polycarbonate plastics and epoxy resins. The applications result in consumer exposure to BPA via the diet. The toxicity and hormonal activity of BPA in laboratory animals have been studied and hormone related cancers and metabolic disorders have been observed in rodents. The objective of this project was to study effects on bone tissues of rats exposed to low doses of BPA *in utero* and during lactation. 110 pregnant Wistar rats were randomly distributed into five groups and gavaged with (0, 0.025, 0.25, 5 or 50 mg BPA/kg body weight (b.w.)/day) from gestation day 7 until weaning at pup day 22, offspring were exposed *in utero* and during lactation. Body weights of the offspring were recorded and right femurs were collected for geometry and densitometry measurements using peripheral quantitative computed tomography and also for analysis of biomechanical properties via three-point bending. The major findings of the study: Increased femur length in female offspring of dams exposed to 0.025 mg BPA/kg b.w./day or 5 mg BPA/kg b.w./day; Increased cortical thickness of male offspring of dams exposed to 0.025 mg BPA/kg b.w./day. No effects of biomechanical properties were seen on the bones of either sex offspring.

In conclusion, this study demonstrates that *in utero* and lactational exposure to BPA at levels relevant to current human exposure alters femoral geometry in rat offspring. BPA showed endocrine disruptive effects on both female and male offspring bone tissue. The mechanisms behind these differences are unknown.

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1. INTRODUCTION

1.1. Bone

Bone is a living mineralized tissue with vital functions in the body. It forms the skeletal system and supports the body weight and movement; bone protects the organs that it surrounds; it stores minerals (*e.g*. calcium and phosphorus) and produces blood cells in the red bone marrow. As a tissue, mature bone consists of extracellular matrix with approximately 35% organic and 65% inorganic material and bone cells (Seeley *et al*. 2003).

The shaft of a long bone, for example a femur, is called diaphysis, which mainly consists of compact bone (cortical bone) and the ends of a long bone are called epiphyses which consist primarily of cancellous bone (trabecular bone) (Figure 1). The area where the diaphysis meets the epiphysis is metaphysis. It includes the epiphyseal plate in growing bones and red marrow, the site of blood cell formation. The open space within the diaphysis is medullary cavity (or marrow cavity) that is filled with yellow marrow containing adipose tissue (Seeley *et al*. 2003).

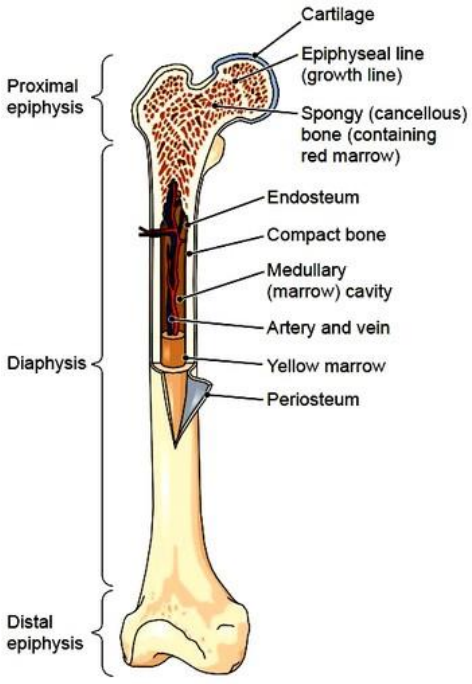

Figure 1. The structure of a femur.

1.1.1. Bone development and growth

In the mammalian embryo and fetus, the skeleton is principally made up of cartilage, a tough and flexible connective tissue that contains no minerals. The formation of cartilage in humans begins at approximately the fourth week of development. As the fetus grows, osteoblasts and osteoclasts slowly replace cartilage cells and bone formation (ossification) begins (Seeley *et al*. 2003). Osteoclasts are multinucleated cells that dissolve both the inorganic and the protein portions of the bone matrix. Osteoblasts differentiate from mesenchymal stem cells and are responsible for the production of new bone matrix. There are mainly two kinds of ossification, intramembranous and endochondral ossification. Intramembranous ossification is a process in which the mesenchymal tissue is converted directly into bone tissue. In humans this occurs primarily in the skull bones and begins at approximately the eighth week of development and is completed by two years of age.

In other cases, mesenchymal cells differentiate into chondrocytes which later stop dividing and increase their volume dramatically, becoming hypertrophic chondrocytes. The hypertrophic chondrocytes die by apoptosis and this space will become bone marrow. As the cartilage cells die, a group of cells that have surrounded the cartilage model differentiate into osteoblasts. The osteoblasts begin forming bone matrix on the partially degraded cartilage. Eventually, all the cartilage is replaced by bone. This process is called endochondral ossification (Gilbert 2006).

During infancy and youth, interstitial growth of the epiphyseal plate cartilage and its replacement by bone enable long bone to lengthen and appositional growth makes all bones grow in width. Long bones increase in width (diameter) when osteoblasts beneath the periosteum secrete bone matrix on the external bone surface and osteoclasts on the endosteal surface of the diaphysis remove bone. Normally, the amount of bone that is removed is slightly less than that is formed so a thicker and stronger bone is produced but the bone is also prevented from becoming too heavy.

Longitudinal bone growth ends when the bone of the epiphysis and diaphysis fuses, which is called epiphyseal plate closure. In humans this happens at about 18 years of age for girls and at about 21 years of age for boys (Marieb *et al*. 2010). In rats the bones continue to grow throughout life, although at a slower rate in adult animals.

1.1.2. Hormonal regulation of bone growth

The growth of bones that occurs until young adulthood is extensively controlled by a symphony of hormones including growth hormone, thyroid hormones and sex hormones (testosterone and estrogen). At the later stage of puberty, estrogen induces epiphyseal plate closure and ends longitudinal bone growth (Marieb *et al*. 2010).

During the second half of the 20th century, estrogen therapy was administered to prevent otherwise healthy girls with tall stature from becoming tall adults by inhibiting further linear growth (Lee *et al*. 2006).

1.2. Endocrine disruptors

Endocrine disruptors (EDs) are exogenous chemicals that interfere with the normal function of hormones by mimicking effects of natural hormones, blocking the synthesis of a hormone, the binding to its receptor and/or interfering with the transport or elimination of a hormone. EDs are highly heterogeneous molecules and include synthetic chemicals and their byproducts *e.g*. polychlorinated biphenyls (PCBs), dioxins, bisphenol A (BPA), phthalates, dichlorodiphenyltrichloroethane (DDT), vinclozolin and diethylstilbestrol (DES) as well as natural chemicals such as phytoestrogens found in human and animal food like soybeans and alfalfa (WHO/UNEP 2013).

1.2.1. Bisphenol A

Bisphenol A (BPA) (CAS Number: 80-05-7, Chemical name: 4. 4'-isopropylidenediphenol) is a synthetic organic chemical largely produced for polycarbonate compound and epoxy resins (Figure 5). Plastics made with polycarbonates containing BPA monomers have a high impact strength, increased hardness and transparency. Polycarbonate plastic is resistant to a wide range of temperatures (between about –40˚C and 145˚C), many acids, greases and oils. Some flame retardants and rubber chemicals also contain BPA (KEMI 2013).

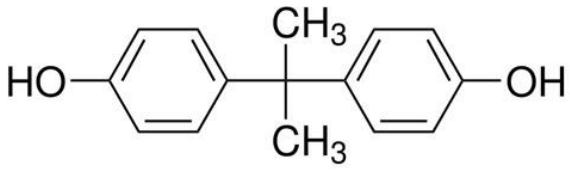

Figure 5. Chemical Structure of bisphenol A (CAS Number: 80-05-7).

BPA has a water solubility of 120 to 300 mg/l at 20 to 25 ˚C (pH 7) and the log octanol-water partition coefficient (Kow) value is between 2.2 and 3.8. The vapor pressure of BPA is 5.32×10^{-7} Pa at 25˚C. Following an oral administration in rodents and humans, BPA is extensively absorbed from the gastrointestinal tract and metabolized in the gut and liver into its primary metabolite monoglucoronide conjugate and other minor metabolites (BPA sulfate and BPA-3,4-quinone) which are excreted via urine and feces (Doerge *et al*. 2010). New findings suggest that BPA is excreted much more via sweat than in urine. In this study, human serum BPA level was found to be 70% lower than it was in sweat samples among the 20 participants (Genuis *et al.* 2012). In the rat, both BPA and the inactive metabolite of BPA (BPA-glucuronide (BPA-GA)) are proved to pass through placenta to the fetus. Furthermore, the rat fetus has the ability to deconjugate BPA-GA into free BPA which causes toxicities. The fetal liver however, has very low capacity to conjugate BPA into its inactive form again (Nishikawa *et al*.2010).

BPA was discovered to be an artificial estrogen already in the 1930s (Dodds *et al*. 1936), long before it was used in production of plastics and resins in 1950s. BPA is used in a multitude of applications and they result in ubiquitous exposure to the general public. Food and beverage packaging lined with epoxy resins containing BPA are considered to be the greatest potential sources. Many reusable plastic bottles and food containers, including infant bottles, are made from BPA-containing polycarbonate plastic. Even though the use of BPA in baby bottles has been banned in some countries, we are still chronically exposed to BPA in the environment through food and drinking water. European Food Safety Authority (EFSA) established the present tolerable daily intake (TDI) of 50 µg/kg body weight (b.w.) (EFSA 2008), which was based on a NOEAL of 5 mg/kg b.w./day in rats (Tyl *et al*. 2002). However, a re-evaluation for a new TDI was first suggested in 2010 after the EFSA panel meeting and then in 2012 after a review on low dose effects of BPA done by the Swedish Chemicals Agency in 2012 (Beronius *et al*. 2012).

Some known effects of BPA as an endocrine disruptor are mainly shown as hormone-related cancers or metabolic disorders. For example in rodents, early exposure to BPA during organogenesis causes alterations in the mammary gland, which renders it more sensitive to estradiol at later developmental stages and more susceptible to neoplasia. BPA disturbs the regulation of adipose tissue deposition and food intake by acting via estrogen receptors in fat cells and brain cells, and further, it also results in increased insulin resistance and secretion as well as decreased glucose tolerance in adult male rodents. BPA exposure to pregnant women decreases glucose tolerance during pregnancy and increased insulin resistance is seen both during pregnancy and four months post-partum. In amphibians BPA has been shown to block thyroid hormone induced metamorphosis (WHO/UNEP 2013).

1.2.2. Effects of bisphenol A on bone

1.2.2.1. Experimental animals

Loss of bone tissue in aromatase knock-out mice (ArKO mice) was prevented in individuals fed a diet supplemented with 0.1% or 1% (w/w) BPA for 5 months (Toda *et al*. 2002). This

study showed that the BMD in BPA exposed individuals increased in a dose-dependent manner. An *in utero* and lactational exposure to 0.01 mg/kg/day BPA caused a 10.3% decrease of femur mechanical strength in female mice (13 weeks of age), but not in males (Pelch *et al*. 2012). In another study, the tibia BMD of OVX rats decreased after a three month dietary exposure to BPA (0.037 mg/kg b.w. /day or 0.37 mg/kg b.w. /day) compared to the unexposed controls (Seidlova-Wuttke *et al*. 2004). No vertebral malformations or adverse effects on skeletal development were observed in fathead minnows (*Pimephales promelas*) exposed to BPA (0.1 to 1.0 µg/L) from egg stage to 25 to 26 day after hatching (Warner *et al*. 2007).

1.2.2.2. Epidemiological studies

The number of studies on effects of BPA on bone tissues in humans is very limited. Urinary BPA levels were not related to bone mineral densities, bone resorption marker N-telopeptide or bone formation parameter osteocalcin in premenopausal women (Zhao *et al*. 2012).

1.2.2.3. In vitro effects

The Tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) were used as markers for osteoclast and osteoblast activities, respectively and were both found to be suppressed significantly by BPA (10^{-5} M) in the cultured scales of goldfish (Suzuki *et al*. 2003). In another study, BPA (10^{-10}- 10^{-6} M) increased ALP activity and enhanced bone mineralization in MC3T3-E1 cells (osteoblast-like cell line) (Kanno *et al*. 2004).

1.3. Objective of this study

This study is a part of a collaboration project between Division of Toxicology and Risk Assessment, National Food Institute in Denmark, Monica Lind at the Department of Medical Sciences-Occupational and Environmental Medicine, Uppsala University, Sweden and Jan Örberg at the Department of Environmental Toxicology, Uppsala University, Sweden. The aim of the original project was to study endocrine disrupting properties and effects of bisphenol A on reproductive parameters in rats. The objective of this part is to study effects on bone tissues of rats exposed to low doses of BPA *in utero* and during lactation.

2. MATERIAL AND METHOD

2.1. Materials

Bisphenol A (purity > 99.5%, CAS no. 80-05-7) and corn oil were purchased from Sigma-Aldrich (Brøndby, Denmark). Corn oil was provided in glass bottles. The solutions were kept in the dark at room temperature and continuously stirred during the dosing period.

2.2. Animals

110 pregnant Wistar rats were housed in pairs until gestation day (GD) 17 and thereafter individually under standard conditions in polysulfone cages with Aspen wood chip bedding (Tapvei, Gentofte, Denmark), Enviro Dri nesting material (Brogaarden, Denmark) and Tapvei Aspen wood shelters (Brogaarden, Denmark). The animal room was maintained with a 12 hour light-dark cycle (light intensity 500 lux, starting at 9 pm), humidity 55% ± 5, temperature at 21 ˚C ± 1˚C and ventilation changing air 10 times per hour. All rats were fed on a standard diet with soy and alfalfa free ALROMIN 1314 (ALTROMIN GmbH, Lage, Germany). Daily individual body weight was recorded from GD 7 for dams and pups were weighed on pup day (PD) 7 and 14. Tap water was provided in polysulfone bottles ad libitum.

2.3. Experimental design

On GD 4, the dams were randomly distributed into five groups (0, 0.025, 0.25, 5 and 50 mg BPA/kg b.w.) with 22 rats of similar body weight per group. BPA was dissolved in corn oil and the rats were gavaged with a stainless probe 1.2 × 80 mm once daily from GD 7 until weaning at PD 22, at a constant volume of 2 ml/kg b.w. The control group was given corn oil. Maximum numbers of pups from each dam included in the study were one female and one male.

2.4. Preparation of bones

At three months of age, the pups were sacrificed and right femurs from 89 male and 93 female were dissected, cleaned and placed in 10 ml centrifuge tubes with Ringer solution (pH 7.4, Tris 0.3 g/l, NaCl 9 g/l, $CaCl_2 \cdot 2H_2O$ 0.24 g/l, KCl 0.4 g/l, 2.05×10^{-3} M HCl) and frozen at -18˚C pending peripheral quantitative computed tomography (pQCT) and biomechanical testing. Bones were placed in a refrigerator (+8˚C) approximately 24 hours before the analyses. For each pQCT analysis, the femur lengths were measured using a slide caliper (accuracy of 0.1 mm) from the proximal end of the femur to the distal end, with the bone parallel to the ruler. The bone was then covered with a piece of 5×5 cm highly pure non-woven compress (OneMed Sverige AB, Spånga, Sweden) moistened with Ringer solution and then placed in a 10 ml centrifuge tube to prevent the femur from drying.

2.5. pQCT (peripheral Quantitative Computed Tomography)

Femur dimension and composition were examined with an X-ray based density measurement machine, pQCT (Stratec XCT Research SA+, Stratec Medizintechnik, Pforzheim, Germany, software version 6.00) at Uppsala University Hospital, Uppsala, Sweden (Figure 6). The machine was calibrated every day before the measurement started with the manufacturer specified reference object (phantom).

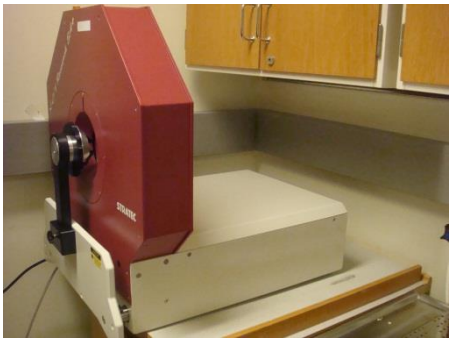

Figure 6. Stratec XCT Reserch SA+ peripheral quantitative computed tomograph (pQCT).

According to the experiment design, the bone was re-positioned between measurements on the pQCT holder so that the scanner could scan across more than 50% of the whole bone length from the distal end of the epiphysis. After a contrast enhancement was generated on the computer screen, the reference line and measurement line(s) were displayed on the scout view (SV) image. A scout view in a CT study is a radiographic image used to plot the locations where the subsequent slice images will be obtained. When the reference line was adjusted to be at the exact edge of the distal epiphysis on the SV, the measurement line(s) were placed accordingly.

2.5.1. Metaphysis measurement

The metaphysis of the femur was analyzed by examining one 1 mm thick slice at 20% of the bone length from the reference line. The following settings were used: voxel size 0.070 mm. peel mode 20, threshold 280 mg/cm^3, contour mode 1 and the measures obtained from the analysis were total bone mineral content (Total BMC, mg/mm), total bone mineral density (Total BMD, mg/mm^3), trabecular bone mineral content (Trabecular BMC, mg/mm), trabecular bone mineral density (Trabecular BMD, mg/mm^3), total cross sectional area (Total CSA, mm^2), trabecular cross sectional area (Trabecular CSA, mm^2) and Periosteal circumference (mm).

2.5.2. Diaphysis measurement

The diaphysis of the femur was analyzed by examining one 1 mm thick slice at 50 % of the bone length from the reference line. The following settings were used: contour mode 1 and threshold 710 mg/ cm^3 and the measures obtained from the analysis were total BMC (mg/mm), total BMD (mg/mm^3), total CSA (mm^2), cortical BMC (mg/mm), cortical BMD (mg/mm^3), cortical CSA (mm^2), cortical thickness (mm), periosteal circumference (mm), endocortical circumference (mm) and marrow cavity (mm^2).

2.5.3. Reproducibility

To create a valid method, the coefficient of variation (CV= Standard deviation × 100 /Mean) was calculated from 10 repeated pQCT measurements performed on a single rat femur. For each measurement, one 1 mm thick slice from the metaphysis and one 1 mm thick from the diaphysis were analyzed.

2.6. Three-point bending test

A three-point bending test was performed to measure the bone strength using an electromechanical material testing machine (Avalon technologies, MN, USA) with a span length of 13 mm and a loading speed of 0.2 mm/sec. The load was applied to the anterior surface of the mid part of the diaphysis with the intention to apply load at the same site as where the pQCT scan had been performed. The load and deformation data were recorded and sampled with 50 Hz. Load at failure (N), displacement at failure (mm), stiffness (slope of the load-deflection curve. representing the elastic deformation, N/mm) and energy absorption until failure (surface under the curve, N×mm) were calculated from the load-deflection curves (Figure 7).

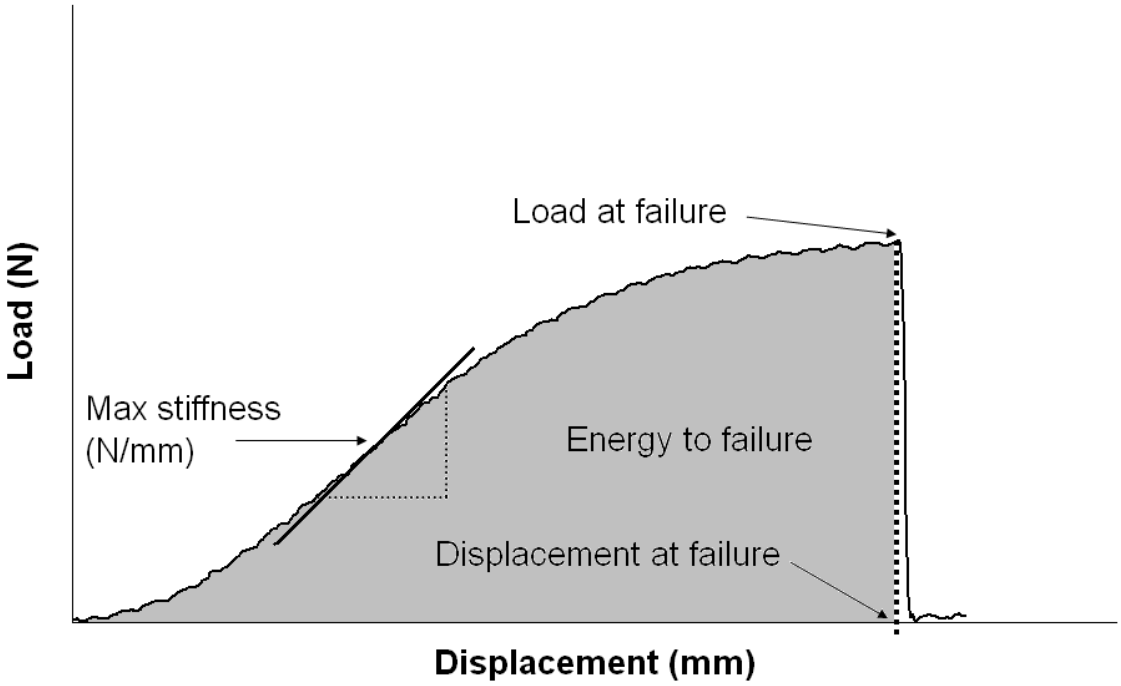

Figure7. A typical three-point bending test results in a curve. Load (N) is a function of Displacement (mm). Max stiffness (N/mm) can be calculated from the slope at the steepest part of the curve. Energy to failure (energy absorption when the bone breaks, N× mm) is the gray area under the curve.

2.7. Statistics

The results were evaluated by analysis of variance (ANOVA) and analysis of covariance (ANCOVA) with Bonferroni/Dunn as the post-hoc test was used to adjust the continuous variable of body weight (StatView, Version 5.0.1; SAS Institute Inc., Cary, NC, USA).

3. RESULTS

3.1. pQCT measurements

3.1.1. Reproducibility

The results from the pQCT reproducibility measurements are presented in tables 1 and 2. 10 repeated pQCT measurements from a single rat femur gave coefficient of variations (CV) that varied from 0.35% to 3.36% at the 20% metaphyseal measure point and 0.22% to 2.74% at the 50% diaphyseal measure point.

Table 1. The reproducibility calculated from 10 repeated measurements conducted on a single rat femur using peripheral quantitative computed tomography (pQCT). The metaphyseal measure point was located at 20% of the total bone length from the distal epiphyseal tip. BMC = bone mineral content, BMD = bone mineral density, CSA = cross sectional area.

Metaphyseal bone 20%	Mean	±	SD	CV(%)
Total BMC (mg/mm)	12.0	±	0.1	0.79
Total BMD (mg/mm^3)	625.4	±	2.2	0.35
Total CSA (mm^2)	19.2	±	0.1	0.73
Trabecular BMC (mg/mm)	1.8	±	0.1	3.36
Trabecular BMD (mg/mm^3)	205.8	±	5.5	2.68
Trabecular CSA (mm^2)	8.6	±	0.1	0.72
Periosteal circumference (mm)	15.5	±	0.1	0.37

Table 2. The reproducibility calculated from 10 repeated measurements conducted on a single rat femur using peripheral quantitative computed tomography (pQCT). The diaphyseal measure point was located at 50% of the total bone length from the distal epiphyseal tip. BMC = bone mineral content, BMD = bone mineral density, CSA = cross sectional area.

Diaphyseal bone 50%	Mean	±	SD	CV(%)
Total BMC (mg/mm)	13.1	±	0.1	0.43
Total BMD (mg/mm^3)	925.0	±	8.1	0.88
Total CSA (mm^2)	14.1	±	0.1	0.97
Cortical BMC (mg/mm)	11.7	±	0.1	0.47
Cortical BMD (mg/mm^3)	1440.9	±	3.2	0.22
Cortical CSA(mm^2)	8.1	±	0.0	0.62
Cortical thinkness (mm)	0.7	±	0.0	1.32
Periosteal circumference (mm)	13.3	±	0.1	0.49
Endocortical circumference (mm)	8.7	±	0.1	1.38
Marrow cavity (mm^2)	6.0	±	0.2	2.74

3.1.2. Measurements

3.1.2.1. Body weight

The mean body weight of the female offspring in the different groups varied between 208.0 and 218.1 g but did not differ significantly (Table 3).

The mean body weight of the male offspring varied between 330.9 and 366.3 g with a significant difference between the treated groups 0.025 and 0.25 mg BPA/kg b.w./day ($p<0.005$) (Table 3). The mean body weight of the male offspring of dams exposed to 0.025 mg BPA/kg b.w./day was 10.7% higher than that of dams exposed to 0.25 mg BPA/kg b.w./day.

3.1.2.2. Bone length

Compared with the control group, the femurs were significantly longer in female offspring of dams exposed to 0.025 or 5 mg BPA/kg b.w./day ($p<0.005$, Figure 8).

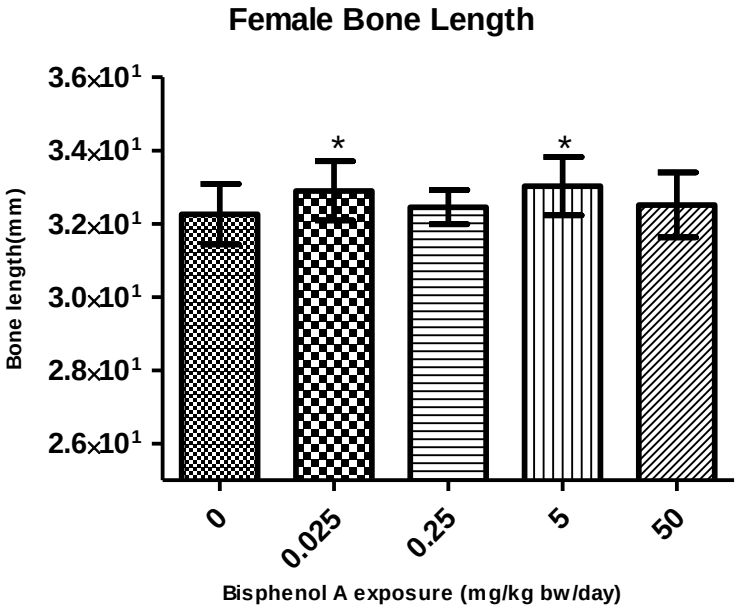

Figure 8. Femur length (mm, mean ± SD, n= 14 to 20) of female Wistar offspring from dams exposed to 0, 0.025, 0.5, 5 or 50 mg BPA/kg b.w./day from gestational day 7 until postnatal day 21. Values with * are statistically significant compared with the control group ($p<0.005$, ANCOVA with Bonferroni post-hoc).

No significant differences were found in femur length between male offspring of dams exposed to BPA and control group. However, the mean femur length from the male offspring of dams exposed to 0.025 mg BPA/kg b.w./day was 3.3% longer ($p<0.005$) compared to those of dams exposed to 0.25 mg BPA/kg b.w./day (Table 3).

Table 3. Body weight (g, mean ± SD) and femur length (mm, mean ± SD) of Wistar offspring from dams exposed to 0, 0.025, 0.5, 5 or 50 mg BPA/kg b.w./day from gestational day 7 until postnatal day 21. The value with * is statistically significant compared with the one of the control group and values with the same letter (a or b) are significantly different from each other ($p<0.005$). n= number of individuals, ANOVA was used for body weight and ANCOVA with Bonferroni post-hoc was used for femur length.

		Bisphenol A (mg/kg body weight/day)				
		0 (n=19)	0.025 (n=21)	0.25 (n=15)	5 (n=18)	50 (n=15)
Female	Body weight (g)	212.7 ± 14.2	218.1 ± 11.8	208.0 ± 14.5	213.9 ± 16.2	219.3 ± 36.9
	Bone length (mm)	32.3 ± 0.8	32.9 ± 0.8 *	32.5 ± 0.5	33.0 ± 0.8 *	32.5 ± 0.9
		0 (n=20)	0.025 (n=20)	0.25 (n=18)	5 (n=20)	50 (n=14)
Male	Body weight (g)	349.5 ± 39.6	366.3 ± 30.7 a	330.9 ± 28.8 a	340.7 ± 32.5	343.3 ± 35.4
	Bone length (mm)	35.7 ± 1.5	36.5 ± 0.9 b	35.3 ± 1.3 b	35.9 ± 1.1	36.2 ± 0.9

3.1.2.3. Metaphyseal measurements

The results obtained from the metaphyseal measurements on bones from female offspring are presented in table 4. No significant differences were found in the bone geometry and densities between female offspring of dams exposed to BPA and female offspring of control dams.

Table 4. Results (mean ± SD) from peripheral quantitative computed tomography of femur metaphysis variables of female Wistar offspring from dams exposed to 0, 0.025, 0.5, 5 or 50 mg BPA/kg b.w./day from gestational day 7 until postnatal day 21. The metaphyseal measure point was located at 20% of the total bone length from the distal epiphyseal tip. n= number of individuals, ANCOVA with Bonferroni post-hoc was used for analyzing. BMC= bone mineral content, BMD = bone mineral density, CSA =cross sectional area.

Female	Bisphenol A (mg/kg body weight/day)				
	0 (n=20)	0.025 (n=20)	0.25 (n=18)	5 (n=20)	50 (n=14)
Total BMC (mg/mm)	11.4 ± 1.4	11.1 ± 0.9	10.8 ± 1.1	11.2 ± 1.1	11.8 ± 1
Total BMD (mg/mm^3)	816.5 ± 62.8	808.7 ± 51.3	803.7 ± 58.3	825.5 ± 52.4	837.8 ± 49.3
Trabecular BMC (mg/mm)	3.3 ± 0.8	3.1 ± 0.6	3.0 ± 0.6	3.1 ± 0.6	3.5 ± 0.5
Trabecular BMD (mg/mm^3)	523.4 ± 99.1	492.5 ± 86.5	492.4 ± 91.6	514.4 ± 78.9	543.5 ± 71.9
Total CSA (mm^2)	13.9 ± 1	13.8 ± 1.1	13.5 ± 0.9	13.5 ± 0.9	14.2 ± 1.2
Trabecular CSA (mm^2)	6.3 ± 0.4	6.2 ± 0.5	6.1 ± 0.4	6.1 ± 0.4	6.4 ± 0.5
Periosteal circumference (mm)	13.2 ± 0.5	13.2 ± 0.5	13 ± 0.4	13 ± 0.4	13.3 ± 0.6

The results obtained from the metaphyseal measurements on bones from male offspring are presented in table 5. No significant differences were found in the bone geometry and densities between male offspring of dams exposed to BPA and male offspring of control dams.

Table 5. Results (mean ± SD) from peripheral quantitative computed tomography of femur metaphysis variables of male Wistar offspring from dams exposed to 0, 0.025, 0.5, 5 or 50 mg BPA/kg b.w./day from gestational day 7 until postnatal day 21. The metaphyseal measure point was located at 20% of the total bone length from the distal epiphyseal tip. n= number of individuals, ANCOVA with Bonferroni post-hoc was used for analyzing. BMC= bone mineral content, BMD = bone mineral density, CSA =cross sectional area.

Male	Bisphenol A (mg/kg body weight/day)				
	0 (n=19)	0.025 (n=21)	0.25 (n=15)	5 (n=18)	50 (n=15)
Total BMC (mg/mm)	12.4 ± 1.5	12.3 ± 1.3	12 ± 1	12.4 ± 1.1	11.9 ± 1.5
Total BMD (mg/mm^3)	705.1 ± 60.5	677 ± 33.9	686 ± 50	690.7 ± 50	667.1 ± 39.8
Trabecular BMC (mg/mm)	3.1 ± 0.9	2.7 ± 0.7	2.7 ± 0.6	2.9 ± 0.6	2.6 ± 0.7
Trabecular BMD (mg/mm^3)	389.4 ± 101.1	328.4 ± 60.1	348.1 ± 80.5	358.6 ± 74.8	315.3 ± 60
Total CSA (mm^2)	17.6 ± 1.3	18.1 ± 1.5	17.6 ± 1.2	18 ± 0.8	17.8 ± 1.7
Trabecular CSA (mm^2)	7.9 ± 0.6	8.1 ± 0.7	7.9 ± 0.5	8.1 ± 0.3	8 ± 0.8
Periosteal circumference (mm)	14.9 ± 0.5	15.1 ± 0.6	14.9 ± 0.5	15 ± 0.3	14.9 ± 0.7

3.1.2.4. Diaphyseal measurements

The results obtained from the diaphyseal measurements on bones from female offspring are presented in table 6. Total BMC was observed to be significantly lower in offspring of dams exposed to 0.25 mg BPA/kg b.w./day than those of dams exposed to 50 mg BPA/kg b.w./day.

Table 6. Results (mean ± SD) from peripheral quantitative computed tomography of femur diaphysis variables of female Wistar offspring from dams exposed to 0, 0.025, 0.5, 5 or 50 mg BPA/kg b.w./day from gestational day 7 until postnatal day 21. The diaphyseal measure point was located at 50% of the total bone length from the distal epiphyseal tip. Values with the same letter 'a' are significantly different from each other ($p<0.005$, ANCOVA with Bonferroni post-hoc). n= number of individuals. BMC= bone mineral content, BMD = bone mineral density, CSA =cross sectional area, marrow cavity = total CSA – cortical CSA.

Female	Bisphenol A (mg/kg body weight/day)				
	0 (n=19)	0.025 (n=21)	0.25 (n=15)	5 (n=18)	50 (n=15)
Total BMC (mg/mm)	7.6 ± 0.5	7.7 ± 0.4	7.4 ± 0.4 a	7.6 ± 0.5	7.8 ± 0.5 a
Total BMD (mg/mm^3)	922.4 ± 45.9	926.8 ± 41.5	922.0 ± 36.2	932.0 ± 32.9	918.3 ± 40.1
Total CSA (mm)	8.3 ± 0.7	8.3 ± 0.6	8.0 ± 0.6	8.2 ± 0.6	8.5 ± 0.8
Cotical BMC (mg/mm)	6.8 ± 0.4	6.8 ± 0.4	6.6 ± 0.4	6.8 ± 0.5	6.9 ± 0.4
Cortical BMD (mg/mm^3)	1385.9 ± 16.3	1389.3 ± 16.3	1380.6 ± 15.9	1393.1 ± 9.7	1382.8 ± 15.0
Cortical CSA (mm)	4.9 ± 0.3	4.9 ± 0.3	4.8 ± 0.3	4.9 ± 0.3	5.0 ± 0.3
Cortical thickness (mm)	0.59 ± 0.03	0.59 ± 0.02	0.58 ± 0.02	0.59 ± 0.03	0.59 ± 0.03
Periosteal circumference (mm)	10.2 ± 0.4	10.2 ± 0.4	10.0 ± 0.4	10.1 ± 0.4	10.3 ± 0.5
Endocortical circumference (mm)	6.5 ± 0.5	6.5 ± 0.4	6.4 ± 0.4	6.4 ± 0.4	6.7 ± 0.5
Marrow Cavity (mm^2)	3.4 ± 0.5	3.4 ± 0.5	3.3 ± 0.4	3.3 ± 0.4	3.5 ± 0.5

The results obtained from the diaphyseal measurements on bones from male offspring are presented in table 7. Cortical thickness was found to be significantly higher in the offspring of dams exposed to 0.025 mg BPA/kg b.w./day compared with those from the control group.

Significant differences were observed in total BMC, cortical BMC, cortical CSA and cortical thickness between offspring of dams exposed to 0.025 mg BPA/kg b.w./day and those of dams exposed to 0.25 mg BPA/kg b.w./day.

Table 7. Results (mean ± SD) from peripheral quantitative computed tomography of femur diaphysis variables of male Wistar offspring from dams exposed to 0, 0.025, 0.5, 5 or 50 mg BPA/kg b.w./day from gestational day 7 until postnatal day 21. The diaphyseal measure point was located at 50% of the total bone length from the distal epiphyseal tip. The value with * is statistically significant compared with the one of the control group and values with the same letter (a, b, c or d) are significantly different from each other ($p<0.005$, ANCOVA with Bonferroni post-hoc). n= number of individuals. BMC= bone mineral content, BMD = bone mineral density, CSA =cross sectional area, marrow cavity = total CSA- cortical CSA.

Male	Bisphenol A (mg/kg body weight/day)				
	0 (n=19)	0.025 (n=21)	0.25 (n=16)	5 (n=18)	50 (n=15)
Total BMC (mg/mm)	9.5 ± 0.9	9.9 ± 0.7 a	9.2 ± 0.7a	9.7 ± 0.6	9.5 ± 0.7
Total BMD (mg/mm^3)	888.9 ± 43.0	918.3 ± 35.5	887.6 ± 24.5	906.4 ± 28.8	893.9 ± 45.8
Total CSA (mm)	10.7 ± 1.0	10.7 ± 0.7	10.4 ± 0.9	10.7 ± 0.8	10.7 ± 1.0
Cotical BMC (mg/mm)	8.4 ± 0.8	8.8 ± 0.6 b	8.2 ± 0.7 b	8.7 ± 0.5	8.5 ± 0.6
Cortical BMD (mg/mm^3)	1372.3 ± 10.0	1380.6 ± 12.4	1372.7 ± 9.6	1376.7 ± 11.2	1376.1 ± 12.7
Cortical CSA (mm)	6.1 ± 0.6	6.4 ± 0.4 c	6.0 ± 0.5 c	6.3 ± 0.4	6.1 ± 0.4
Cortical thickness (mm)	0.64 ± 0.05	0.67 ± 0.04* d	0.63 ± 0.03 d	0.66 ± 0.03	0.64 ± 0.04
Periosteal circumference (mm)	11.6 ± 0.5	11.6 ± 0.4	11.4 ± 0.5	11.6 ± 0.4	11.6 ± 0.5
Endocortical circumference (mm)	7.5 ± 0.5	7.4 ± 0.4	7.5 ± 0.4	7.5 ± 0.4	7.5 ± 0.6
Marrow Cavity (mm^2)	4.5 ± 0.6	4.4 ± 0.5	4.4 ± 0.5	4.4 ± 0.5	4.5 ± 0.7

3.2. Three- point bending test

The results from the three- point bending test are presented in tables 8 and 9. No significant differences in bone strength were observed between control and treated groups in neither female nor male offspring.

Table 8. Results (mean ± SD) from three-point bending test performed on femurs of female Wistar offspring from dams exposed to 0, 0.025, 0.5, 5 or 50 mg BPA/kg b.w./day from gestational day 7 until postnatal day 21. The test was performed using an electromechanical material testing machine (Avalon technologies, MN, USA). The load was applied at the mid-diaphyseal pQCT measure point (at about 50% of the total bone length from the distal tip) with a loading speed of 0.2mm/sec. Analysis was done by using ANCOVA with Bonferroni post-hoc. No significant differences in bone strength were found unless p-value is less than 0.005. n= number of individuals.

Female	Bisphenol A (mg/kg body weight/day)				
	0 (n=20)	0.025 (n=20)	0.25 (n=18)	5 (n=20)	50 (n=14)
Displacement (mm)	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.2
Load at failure (N)	106.1 ± 8.4	108.2 ± 10.2	103.8 ± 9.0	108.1 ± 12.1	111.0 ± 12.5
Max Stiffness (N/mm)	182.1 ± 23.6	176.9 ± 29.2	162.6 ± 28.1	176.2 ± 28.5	185.8 ± 28.1
Energy (N×mm)	75.1 ± 17.2	79.6 ± 19.8	70.9 ± 11.0	72.1 ± 11.1	78.6 ± 19.3

Table 9. Results (mean ± SD) from three-point bending test performed on femurs of male Wistar offspring from dams exposed to 0, 0.025, 0.5, 5 or 50 mg BPA/kg b.w./day from gestational day 7 until postnatal day 21. The test was performed using an electromechanical material testing machine (Avalon technologies, MN, USA). The load was applied at the mid-diaphyseal pQCT measure point (at about 50% of the total bone length from the distal tip) with a loading speed of 0.2mm/sec. Analysis was done by using ANCOVA with Bonferroni post-hoc. No significant differences in bone strength were found unless p-value is less than 0.005. n= number of individuals.

Male	Bisphenol A (mg/kg body weight/day)				
	0(n=19)	0.025 (n=21)	0.25(n=15)	5(n=18)	50 (n=15)
Displacement (mm)	1.6 ± 0.3	1.5 ± 0.2	1.5 ± 0.2	1.4 ± 0.2	1.6 ± 0.3
Load at failure (N)	131.6 ± 14.0	141.9 ± 13.8	130.9 ± 15.9	137.2 ± 13.5	133.9 ± 13.5
Max Stiffness (N/mm)	220.4 ± 23.2	227.8 ± 29.7	211.8 ± 36.4	222.0 ± 18.8	223.3 ± 29.2
Engergy (N×mm)	147.9 ± 39.0	151.0 ± 24.6	144.2 ± 31.5	139.3 ± 27.6	148.6 ± 40.2

4. DISCUSSION

The most interesting result of this study was that the femur length of female offspring of dams exposed to 0.025 mg BPA/kg b.w./day or 5 mg BPA/kg b.w./day was significantly longer compared to that of dams from the control group. The exposure period was chosen to cover the most sensitive periods of the development of the rat reproductive system. The BPA doses used, with the lowest observed adverse effect level (50 mg/kg b.w./day) in rats (Tyl *et al*. 2002) as the highest exposure level, are hence considered as low doses. The reproducibility test provided coefficient of variations between 0.22% and 3.36% for the pQCT methodology which ensured the quality of the test.

When the offspring was evaluated in adulthood, no differences in body weight were found in females. However, the femur length of female offspring of dams exposed to 0.025 mg BPA/kg b.w./day or 5 mg BPA/kg b.w./day was significantly longer compared to that of dams from the control group with a 1.9% and 2.2% increase, respectively ($p<0.005$). As estrogens promote the fusion of the growth plate, this finding might indicate an anti-estrogenic effect of BPA on bone tissue in female offspring (Kennedy *et al*.1999; Nilsson *et al*. 2001; Weise 2001).

The cortical thickness was significantly greater (4.7%) in male offspring of dams exposed to 0.025 mg BPA/kg b.w./day compared to those of dams from the control group. A possible explanation to this effect is the slight decrease of endocortical circumference. The femur periosteal circumferences (mean ± SD) in offspring of control dams and in offspring of dams exposed to 0.025 mg BPA/kg b.w./day were 11.6 ± 0.5 and 11.6 ± 0.4 mm, respectively, and the endocortical circumference (mean ± SD) were 7.5 ± 0.5 and 7.4 ± 0.4 mm, respectively (Figure 9). In both sexes, estrogen decreases the endocortical resorption of the diaphysis and thereby causing an increased cortical thickness (Almeida *et al*. 2013).

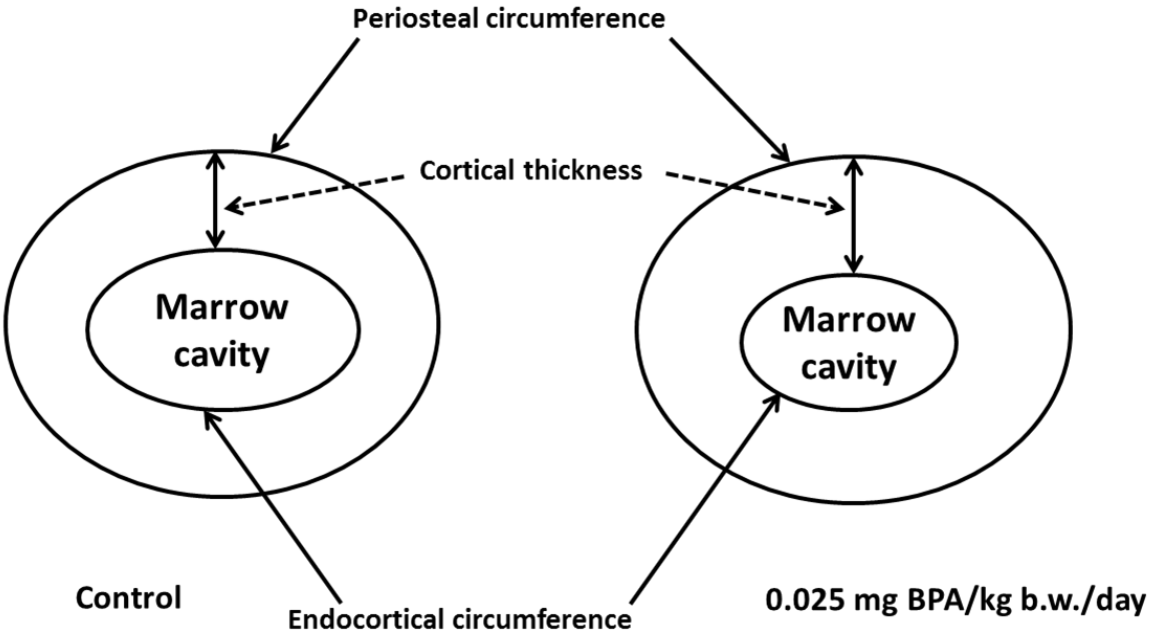

Figure 9. Cross sectional dimension of the femur showing the increase in cortical thickness of male Wistar offspring of dams exposed to 0.025 mg BPA/kg b.w./day from gestational day 7 until postnatal day compared to those of control. The diameters of periosteal circumferences were the same in both groups of femurs whereas the diameter of the endocortical circumference was smaller in the treated femurs compared to that in the controls (The illustration depicts the cortical thickness increase and does not represent the actual sizes of circumferences).

The body weight was 10.7% lower and the bone length was 3.3% shorter in male offspring of dams exposed to 0.25 mg BPA/kg b.w./day compared to those of dams exposed to 0.025 mg BPA/kg b.w./day (Table 3). From the pQCT results, the variables total BMC, cortical BMC, cortical CSA and cortical thickness from the male offspring of dams exposed to 0.25 mg BPA/kg b.w./day were all seen to be significantly lower than the male offspring of dams exposed to 0.025 mg BPA/kg b.w./day. The mechanisms behind all these differences are not known.

No change in the bone strength could be seen from the biomechanical test of this study. However, a torsional loading test by Pelch *et al.* (2012) proved that a developmental

exposure to BPA (0.01 mg/kg b.w./day) resulted in a decrease of femur energy to failure in female mice.

In conclusion, this study demonstrates that *in utero* and lactational exposure to BPA at levels relevant to current human exposure alters femoral geometry in rat offspring. BPA showed endocrine disruptive effects on both female and male offspring bone tissue.

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