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Sex-specific effects of low-dose gestational estradiol-17β exposure on bone development in porcine offspring

Veronika L. Flöter[a,b], Gabriela Galateanu[c], Rainer W. Fürst[a], Dana Seidlová-Wuttke[d], Wolfgang Wuttke[d], Erich Möstl[e], Thomas B. Hildebrandt[c], Susanne E. Ulbrich[a,*]

[a] Physiology Weihenstephan, Technische Universität München, Weihenstephaner Berg 3, 85354 Freising, Germany
[b] Z I E L PhD Graduate School 'Nutritional Adaptation and Epigenetic Mechanisms', Technische Universität München, Freising, Germany
[c] Department of Reproduction Management, Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Straße 17, 10315 Berlin, Germany
[d] Department of Endocrinology, University Medicine Goettingen, Robert-Koch-Str. 40, 37099 Goettingen, Germany
[e] Department of Biomedical Sciences, University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210 Vienna, Austria

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ABSTRACT

Estrogens are important for the bone development and health. Exposure to endocrine disrupting chemicals during the early development has been shown to affect the bone phenotype later in life. Several studies have been performed in rodents, while in larger animals that are important to bridge the gap to humans there is a paucity of data. To this end, the pig as large animal model was used in the present study to assess the influence of gestational estradiol-17β (E2) exposure on the bone development of the prepubertal and adult offspring. Two low doses (0.05 and 10 μg E2/kg body weight) referring to the 'acceptable daily intake' (ADI) and the 'no observed effect level' (NOEL) as stated for humans, and a high-dose (1000 μg E2/kg body weight), respectively, were fed to the sows every day from insemination until delivery. In the male prepubertal offspring, the ADI dose group had a lower strength strain index ($p = 0.002$) at the proximal tibia compared to controls, which was determined by peripheral quantitative computed tomography. Prepubertal females were not significantly affected. However, there was a higher cortical cross-sectional area (CSA) ($p = 0.03$) and total CSA ($p = 0.02$) at the femur midpoint in the adult female offspring of the NOEL dose group as measured by computed tomography. These effects were independent from plasma hormone concentrations (leptin, IGF1, estrogens), which remained unaltered. Overall, sex-specific effects on bone development and non-monotonic dose responses were observed. These results substantiate the high sensitivity of developing organisms to exogenous estrogens.

1. Introduction

Humans and animals are ubiquitously exposed to natural and synthetic substances with estrogenic activity potentially acting as endocrine disrupting chemicals (EDCs) on various organs and body systems (Diamanti-Kandarakis et al., 2009; McLachlan, 2001). According to the Developmental Origin of Health and Disease hypothesis (DOHaD), developmental plasticity allows early environmental changes to result in epigenetic adaptations possibly affecting the onset of diseases during adult life (Hochberg et al., 2011). In this regard, prenatal and early postnatal phases have been demonstrated as sensitive to exogenous influences. Developing organisms can strongly respond to even very low doses of estrogenic EDCs, as endogenous hormone levels are low while their receptors are already in place (Aksglaede et al., 2006; Barle et al., 2008; Knapczyk et al., 2008; McLachlan, 2001; Nilsson et al., 2002). Such effects have also been shown for natural substances such as estradiol-17β (E2) (Fürst et al., 2012; Rasier et al., 2006).

Consequently, studies analyzing early EDC exposure on bone development and metabolism, which have mainly been conducted in rodents, demonstrated various direct and/or lasting effects (Agas

Abbreviations: ADI, acceptable daily intake; BMD, bone mineral density; CT, computed tomography; CL, corpus luteum; CSA, cross-sectional area; DES, diethylstilbestrol; EDC, endocrine disrupting chemical; EIA, enzyme immunoassay; E2, estradiol-17β; ER, estrogen receptor; HU, Hounsfield units; NOEL, no observed effect level; pQCT, peripheral quantitative computed tomography; SSI, strength strain index.

* Corresponding author. Current address: ETH Zurich, Animal Physiology, Institute of Agricultural Sciences, Universitätstr. 2, 8092 Zurich, Switzerland.
E-mail addresses: pistek@wzw.tum.de (V.L. Flöter), galateanu@izw-berlin.de (G. Galateanu), rainer.fuerst@helmholtz-muenchen.de (R.W. Fürst), d.swuttke@verdevital.de (D. Seidlová-Wuttke), w.wuttke@verdevital.de (W. Wuttke), Erich.Moestl@vetmeduni.ac.at (E. Möstl), hildebrandt@izw-berlin.de (T.B. Hildebrandt), seu@ethz.ch (S.E. Ulbrich).

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et al., 2013). The outcome can depend on the time of exposure, the substance and dosage, the species or strain, the gender, the time of analysis, as well as the bone and bone area under investigation. For example, *in utero* and neonatal EDC exposure have often shown to result in an increased bone mineral density (BMD) in adult female rodent offspring (Kaludjerovic and Ward, 2008, 2009; Piekarz and Ward, 2007; Rowas et al., 2012), while the results are more dissimilar in large animals (Hermsen et al., 2008; Lundberg et al., 2006). Conflicting results have also been described regarding BMD in male offspring (Hermsen et al., 2008; Kaludjerovic and Ward, 2008; Lind et al., 2009; Piekarz and Ward, 2007; Rowas et al., 2012 Lind et al., 2009; Piekarz and Ward, 2007; Rowas et al., 2012). Furthermore, studies on female rodent offspring depicted an increase in the peak load (Kaludjerovic and Ward, 2008; Piekarz and Ward, 2007 Piekarz and Ward, 2007), whereas a reduction in bone strength parameters and an increased femur length have also been shown (Pelch et al., 2012). The latter study by Pelch and colleagues (Pelch et al., 2012) specifically used a low-dose diethylstilbestrol (DES) treatment, which was applied during gestation and lactation. In contrast, Migliacchio and colleagues (Migliaccio et al., 1996) showed that the same low-dose given only during gestation did not alter femur length and led to an increase in bone mass. Similar to the effects on the bone length, strength and density, alterations have also been described concerning several bone area parameters (Hermsen et al., 2008; Kaludjerovic and Ward, 2008; Lundberg et al., 2006; Pelch et al., 2012 Lundberg et al., 2006; Pelch et al., 2012; Rowas et al., 2012). In female rhesus monkeys, an increased total cross-sectional area (CSA) at the femur midpoint was demonstrated in a low-dose but not in a high-dose treatment group (Hermsen et al., 2008).

Lasting alterations in the bone phenotype may be due to direct influences of EDCs on bone cell number and activity (Agas et al., 2013; Chen et al., 2009; Hochberg et al., 2011; Javaid and Cooper, 2002). In addition, EDCs could indirectly affect the bone development through alterations of endocrine functions (Agas et al., 2013; Lundberg et al., 2006; Rasier et al., 2006), the onset of puberty (Bonjour and Chevalley, 2014; Rasier et al., 2006 Rasier et al., 2006) and/or body fat content (Csakvary et al., 2012; Fürst et al., 2012; Zhuo et al., 2014). One mechanism causing lasting changes is the alteration of epigenetic marks such as DNA methylation (Hochberg et al., 2011; McLachlan, 2001; Nilsson and Skinner, 2015). In line with this, steroid hormone receptor complexes are able to change histone modifications (Wierman, 2007).

In addition to rodents, the use of large animal models is important to transfer experimental results to humans. The pig has been used in bone research, since human bones are closely resembled (FDA, 1994; Litten-Brown et al., 2010; Pearce et al., 2007 Litten-Brown et al., 2010; Pearce et al., 2007). Consistently, the U.S. Food and Drug Administration (FDA) has recommended to use larger animals such as the pig next to ovariectomized rats as second species for preclinical drug evaluation for treating postmenopausal osteoporosis (FDA, 1994). However, there are only a few studies using large animals to analyze the influences of EDC exposure during gestation and/or neonatal on the bone development (Agas et al., 2013; Gutleb et al., 2010; Hermsen et al., 2008; Lind et al., 2009, 2010; Lundberg et al., 2006). The published results indicate some effects, although in parts deviating from data using rodents. A reason could be that – similar to humans – in large animal models higher estrogen concentrations prevail during pregnancy compared to rodents, due to placental estrogen synthesis, which is absent in the latter (Challis and Linzell, 1971; Lange et al., 2002; Robertson and King, 1974; Witorsch, 2002).

In this study, effects of gestational E2 exposure on the bone development and associated endocrine parameters were analyzed in porcine offspring. Since hormones can show non-monotonic dose responses with some effects specifically occurring at low doses (Vandenberg et al., 2012), the focus was laid on two low doses corresponding to the safety thresholds for humans—the acceptable daily intake (ADI) and the no observed effect level (NOEL) (JECFA, 1999). To our knowledge, this is the first study addressing early estrogen exposure on long term bone outcome in pigs.

2. Materials and methods

2.1. Animal experiment

The animal trial was conducted as described earlier (Fürst et al., 2012; Pistek et al., 2013). In brief, sows (n = 6–7/group) were orally exposed to E2 (1, 3, 5(10)-ESTRATRIEN-3, 17β-DIOL, Steraloids, Newport. USA) twice daily (0, 0.05, 10 and 1000 μg E2 per kg body weight (BW) per day (d), respectively) from insemination until delivery. At birth, no significant differences of the analyzed parameters, including the numbers of piglets, their weight and gender distribution, were detected (Fürst et al., 2012). Male and female offspring (n = 10–12 per group, overall n = 42 and 46, respectively) were slaughtered prepubertally at the age of 8 weeks (d 56) and 9 weeks (d 63), respectively. A second group of females (n = 7–13/group; overall n = 41) was kept until the age of about one year. Siblings were included in the experiments; on average two per sow. Adult boars were not assessed due to housing limitations. Starting when these gilts were 23 weeks old, a fresh rectal feces sample was taken each week to detect the first corpus luteum (CL) formation as a marker of puberty. These samples were immediately put on ice and stored at −20 °C. Prior to slaughtering, estrous cycle behavior was monitored at least once a day and the animals were slaughtered during the luteal phase (d 10 to d 13 post estrus) after at least three estrous cycles.

The femur and tibia from the right hind leg of all animals were stored at −20 °C, after they were separated from most of the surrounding tissue. Plasma was obtained from EDTA (AppliChem, Darmstadt, Germany) supplemented blood after centrifugation at 4 °C and was then stored at −20 °C.

The animal trial was approved by the District Government of Upper Bavaria and performed in accordance with accepted standards of humane animal care.

2.2. Bone measurements

Both the femur and the tibia of the prepubertal male and female offspring were analyzed using peripheral quantitative computed tomography (pQCT, STRATEC XCT 2000 (SA); Stratec, Pforzheim, Germany). The remaining flesh was removed from thawed bones and both the tibia and the femur were separated. Subsequently, pQCT measurements were taken at three bone areas, namely directly below the epiphyseal plate at the proximal tibia and the distal femur, as well as directly above the epiphyseal plate at the proximal femur. By means of a coronal computed radiography (scout view) the scanner was positioned at the site of measurement where three consecutive slices with 1 mm thickness were scanned. Further processing of the data was performed using the software version 5.40 with contour mode 1 and peel mode 2. A lower threshold of 280 mg/cm^3 and an upper threshold of 400 mg/cm^3 were set for the detection of trabecular bone and in order to separate it from the cortical/subcortical region. The threshold for the strength strain index (SSI) was set to 380 mg/cm^3. A voxel size of 0.200 mm was used. Thus, total and trabecular BMD and CSA, as well as the polar SSI were obtained. Cortical bone was still scarce at the time of analysis and was therefore not analyzed. In addition,