DECREASED BONE STRENGTH AFTER IN UTERO / LACTATIONAL TCDD EXPOSURE: TIME-COURSE OF EFFECTS

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Introduction
Developmental defects of the male reproductive system observed in TCDD-treated experimental animals form the basis of the current risk assessment of dioxinlike compounds[1,2]. Recent studies have identified developing teeth and bone as new targets of dioxin toxicity that exhibit similar sensitivity with the developing reproductive system[3,4,5]. This paper is a preliminary report of a study that aims at further characterization of the mechanisms of developmental bone toxicity by defining the time-courses and dose-responses of bone strength in relation with effects on a range of parameters that play a role in bone development and maintenance.

Materials and Methods
Groups of 6-8 pregnant Sprague-Dawley rats were exposed to a single intragastric dose of TCDD on gestational day (GD) 11, two days before the first signs of bone development are observed in fetuses. The maternal dose-levels were 0, 0.03, 0.1, 0.3 and 1.0 ug/kg. Litter sizes were adjusted to 4 males + 4 females on postnatal day (PND) 1. One male and one female offspring from each litter were sampled on PND 7, 35, 70 and 365. In addition, maternal and fetal samples were collected on GD 19 from groups of 6 dams exposed to 0, 0.3 or 1.0 μg/kg. Plasma and bone samples were collected and preserved for further analysis.

Mechanical properties of the tibial diaphyses were tested with a three-point bending test, and the breaking force and stiffness defined. Biomechanical testing could be carried out only for rats sampled on PND 35 or later. The following hormones were analyzed from plasma of the dams on GD 19 and/or of the offspring at different sampling time points: corticosterone, estradiol (E2), testosterone, progesterone, thyroxin (T4), luteinizing hormone (LH) and follicle stimulating hormone (FSH) using radioimmunoassay (RIA) or two-site time-resolved immunofluorometric assay (DELFIA; for corticosterone, LH and FSH). For gene expression studies humerus was homogenized and RNA isolated using GenElute Mammalian Total RNA kit (Sigma-Aldrich) combined with Turbo DNA-free DNase treatment (Ambion). cDNA was generated by Omniscript RT Kit (Qiagen) and used as a template for quantitative PCR analysis. mRNA concentrations of RUNX2, alkaline phosphatase and osteocalcin were analyzed using SYBR Green PCR Master Mix and Applied Biosystems 7000 Real-Time PCR System (Applied Biosystems). Standard curves were generated using isolated and purified PCR products produced with the same primers designed for quantitative PCR. The expression levels were related to mRNA concentrations of house keeping gene GAPDH to normalize the amount of cDNA in PCR reactions.

Results and Discussion
TCDD treatment did not affect the maternal body weight or body weight gain. Body weight of female fetuses on GD 19 was not affected, but that of male fetuses was slightly but significantly decreased. Body weight of both male and female offspring were below controls on PND 7 and 35, but not thereafter. The length of the long bones (femur, tibia, humerus) and the number of ossification centres in sternum were slightly but nonsignificantly decreased in TCDD-treated fetuses on GD 19. The decrease in tibial length was significant on PND 35 and 70 at 1.0 ug/kg TCDD and on PND 70 females also at 0.1, 0.3 and 1.0 ug/kg, but the difference in bone length had disappeared by PND 365.

Tibial breaking force and stiffness showed a dose-dependent decrease on PND 35 and 70 (Fig. 1.), and significant decreases were observed at 0.1-1.0 ug/kg TCDD. Similar decreases have been observed earlier after adult[6] and *in utero* / lactational[4] TCDD exposure, but not at dose-levels below 1.0 ug/kg.

On GD 19 maternal plasma corticosterone, E2, progesterone and T4 levels were not affected by the treatment, but FSH and LH levels were slightly decreased at 1.0 ug/kg. In male fetuses plasma LH was decreased to 25% of the control value at 1.0 ug/kg TCDD, and corticosterone showed an dose-dependently increasing trend, but the difference was not statistically significant. In female fetuses plasma progesterone levels were dose-dependently increased. Plasma T4 levels were not affected in the offspring on GD 19 and PND 7, but they were significantly decreased on PND 35, most likely due to increased TCDD exposure during lactation.

Quantitative RT-PCR analysis on humerus collected on PND 7, 35 and 70 revealed no significant changes in the expression levels of CYP1A1, osteocalcin, runt-related transcription factor 2 (RUNX2, also called cbfa1) or estrogen receptor beta (ERbeta).

In conclusion, *in utero* / lactational TCDD treatment resulted in dose-related decrease in bone strength, but it seems unlikely that the alterations observed in circulating levels of hormones analyzed so far would play a major causative role in the decreased bone strength.

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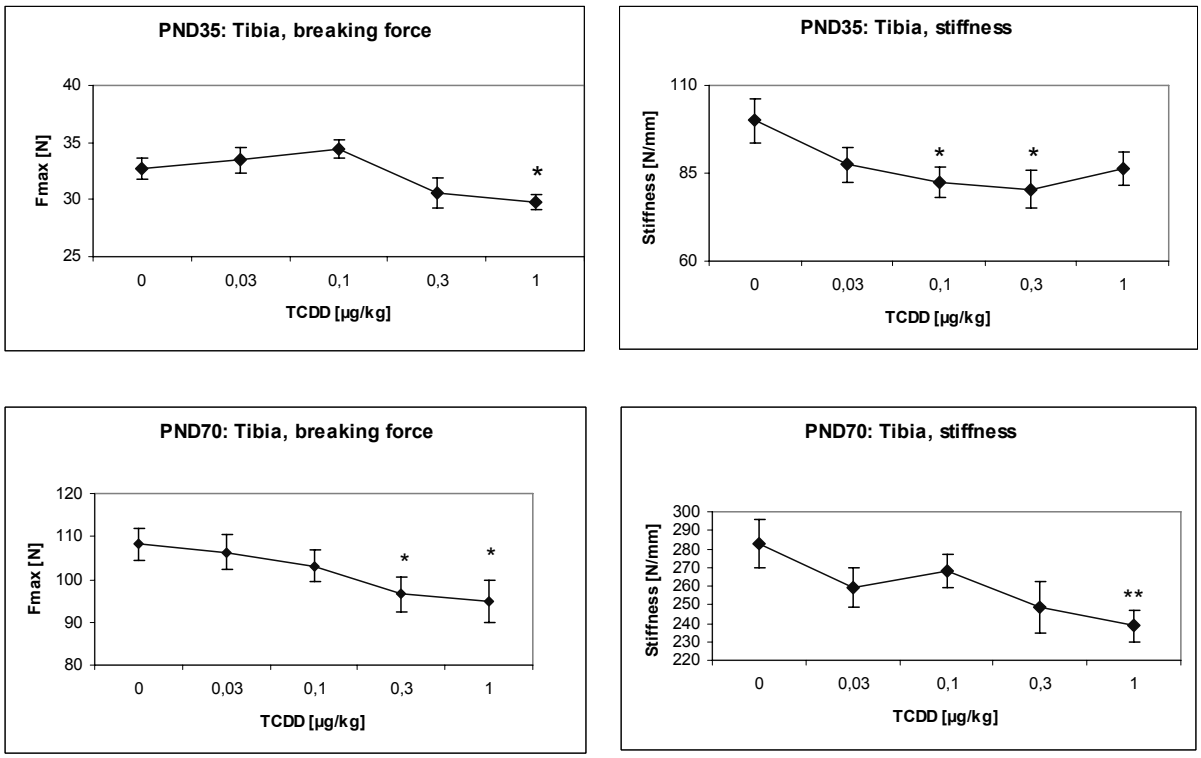

Figure 1. Dose-responses for tibial breaking force (right panel) and stiffness (left panel) of rat offspring exposed to TCDD *in utero* / lactationally on postnatal days 35 (upper graph) and 70 (lower graph), mean ± SE, n=12-16. Statistics: *p<0.05, **p<0.01.