Effects of combined in *utero* and lactational exposure of 2,3,7,8-tetrachlorodibenzo-p-dioxin on early development of bone

Sami Kauppinen

Kandidaatin tutkielma

Hyvinvointitekniikan tutkinto-ohjelma

Lääketieteen tekniikka

Oulun yliopisto

2017

Kauppinen Sami, Faculty of Medicine, University of Oulu, Bachelor thesis, 23 pages.

Abstract

Introduction: Dioxins are environmental contaminants belonging to a group known as persistent organic pollutants (POPs). Dioxins as many other POP's have been identified as endocrine disruptors and they have various systemic effects. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the most potent toxin of the dioxin family and it is known to affect bone tissue through aryl hydrocarbon receptor. Aim is to study dose dependent effects of maternal TCDD exposure on early bone development in offspring.

Methods: TCDD was mixed in corn oil and a single weight adjusted dose of TCDD was administrated to pregnant Sprague-Dawley rats on 11th gestational day. This resulted in following groups: Control group: 0µg/kg bw, Group 1: 0,03µg/kg bw, Group 2: 0,1µg/kg bw, Group 3: 0,3µg/kg bw, Group 4: 1,0µg/kg bw. Offspring was at postnatal day (PND) 7 and bone samples were harvested for imaged with µCT analyses.

Results: Tibias showed decreases in parameters defining bone morphology in both sexes especially with low doses. Male tibias were also associated with a smaller overall cross-sectional size in the highest dose. Femurs had only minor changes in both sexes.

Conclusion: In conclusion, there are significant changes with the low doses (0.03-0.3µg/kg) and with the high dose (1µg/kg) in both female and male tibias. Changes in bone morphology and geometry indicate of disturbed bone development, both of which have a direct implication on biomechanical properties.

Keywords: Dioxin, TCDD, bone, AHR, µCT

Kauppinen Sami, Lääketieteellinen tiedekunta, Oulun yliopisto, Kandidaatin tutkielma, 23 sivua.

Tiivistelmä

Johdanto: Dioksiinit kuuluvat pysyvien orgaanisten ympäristömyrkkyjen ryhmään. Kuten monet muutkin pysyvät ympäristömyrkyt, dioksiinit häiritsevät hormonitoimintaa ja niillä laaja kirjo systemaattisia vaikutuksia. 2, 3, 7, 8-Tetraklooridibentso-p-dioksiini (TCDD) on tämän ryhmän potentein ympäristömyrkky, jonka tiedetään vaikuttavan luun kehitykseen ja homeostaasiin aryylihiilivetyreseptorin kautta**.** Tutkimuksen tarkoituksena on selvittää miten kehityksen aikana saatu TCDD alistus vaikuttaa luun mikrorakenteeseen eri annoksilla.

Menetelmät: Raskaana olevia Sprague.Dawley rottia altistettiin TCDD -kerta-annoksella 11 päivää hedelmöityksen jälkeen. Tutkimuksessa käytettiin viittä eri TCDD-altistustasoa, jotka annettiin normalisoituina painon mukaan. Altistus annettiin kerta-annoksena suun kautta, jolloin saatiin seuraavat ryhmät: 0µg/kg, Ryhmä 1: 0,03µg/kg, Ryhmä 2: 0,1µg/kg, Ryhmä 3: 0,3µg/kg, Ryhmä 4: 1,0µg/kg. Poikaset (38N, 37U) lopetettiin seitsemän päivää syntymän jälkeen, jonka jälkeen reisi- ja sääriluu kuvattiin mikro-tietokonetomografialaitteella mikrorakenneanalyysejä varten.

Tulokset: Molemmissa sukupuolissa sääriluiden morfologiaa kuvaavilla parametreilla oli laskua verrattuna kontrolliryhmään. Reisiluun parametreista naarailla ja uroksilla löytyi vain pieniä muutoksia.

Johtopäätökset: Jopa erittäin pienet annokset häiritsevät luun kehitystä. Ilmiö oli selvä etenkin sääriluissa, mutta ei niinkään reisiluissa. Muutokset luun morfologiassa ja geometriassa kehityksen varhaisessa vaiheessa voivat johtua luun kasvun hidastumisesta tai muista kasvuhäiriöistä.

Avainsanat: Dioksiini, TCDD, Luu, AHR, Mikro-tietokonetomografia

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1 Introduction

Dioxins are environmental contaminants belonging to a group known as persistent organic pollutants (POPs). Dioxins as many other POP's have been identified as endocrine disruptors and they have various systemic effects such as lowered immune system, altered liver enzyme function and carcinogenetic and reproductive disorders (Guo 2007, Hermsen et al. 2008). Dioxins are found in both human tissue and the environment because they are very persistent. There is no effective xenobiotic metabolic mechanism to eliminate and remove these chemicals. Persistency allows these compounds to accumulate in the food chain and due to lipofilicity they are magnified in fat tissues. Mother's milk is high in fat and thus the dioxin burden in offspring is high.(Hermsen et al. 2008)

The main reason for TCDD having so many effects on many different species is because it is an exogenous ligand with affinity to aryl hydrocarbon receptor (AHR) (Herlin 2013)

1.1 TCDD

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the most potent toxin of the dioxin family. WHO (world health organization) has given TCDD a TEF (toxic equivalency factor) –value of 1, which is the highest value in the TEFs for human risk assessment. For humans a tolerable daily intake is 10 pg/kg bw (van Leeuwen et al. 2000) TCDD half-life in humans has been shown to be between 1.6 and 7 years. Half-lifes in humans depend on the age of the person and the level of exposure to TCDD.(Furness & Whelan 2009)

Basically, there are three ways for a human being to be exposed to TCDD. Occupational exposure, accidental exposure and exposure through food intake. Because TCDD is by-product in some chemical processes and waste disposal, workers at those places can get exposed to quite high levels of TCDD.(van Leeuwen et al. 2000) There have been major incidents causing high exposures. One of these happened in Seveso, Italy in 1976, where an explosion at a chemical plant released dangerous amounts of TCDD. The worst effects of the

explosion were seen in Seveso but other surrounding towns were also affected. Immediate signs of high exposure to TCDD were chloracne and skin lesions. Cancer mortality rate from different cancers after 15 years of the accident was increased to a rate ratio of 1.3 when compared to earlier rate of cancer mortality (Bertazzi 2001).

Food intake is the most common source for mammalian dioxin exposure. TCDD however is luckily not the most common chemical compound hiding in plain sight. TCDD is a model compound used in dioxin toxicology, because it is the most potent of the dioxin family. Therefore, it can be used to assess the risks of less potent dioxins and planar polychlorinated biphenyls and -furans (van Leeuwen et al. 2000) by assuming linear dose dependency.

1.2 TCDD effects on bone

Bone matrix is renewed constantly in balance with osteoclasts and osteoblasts where osteoclasts resorb the old bone matrix and osteoblasts create new bone matrix. TCDD has been shown to affect differentiation of osteoclasts and osteoblasts in vitro, thus decreasing bone formation and increasing bone resorption. (Korkalainen 2009, Koskela et al. 2012) This has been also seen to increase resorption and decrease remodeling in-vivo (Herlin 2013) and causing changes in material properties (Finnilä et al. 2010). A TCDD induced retardation of bone matrix maturation in rats has also been shown.(Finnilä et al. 2010) It is also shown that TCDD up-regulates the active form of vitamin D which leads to decrease in osteoblast activity (Nishimura et al. 2009). In an earlier study it was shown that TCDD exposure in rats altered the cortical bone mechanical properties by making it less resistant to forces in three point bending tests with decreased maximal breaking force and the ability to store energy. Cortical bone morphology was also affected with results such as decreased cortical thickness and cortical porosity and increased tissue mineral density.(Herlin 2013). It has been also shown that a high dose of TCDD (50µg/kg) can affect the bone in as short period as five days (Lind et al. 2009).

Many developmental effects have been observed at low levels of exposure, which suggests a higher sensitivity for a developing structure or organ (Miettinen et al.

2005). For this reason, World Health Organisation has considered developmental defects to be the most critical end points, when assessing risks of dioxin effects for humans (van Leeuwen et al. 2000).

1.3 Aryl hydrocarbon receptor

The Aryl hydrocarbon receptor (AHR) found in humans and other species also is a signal regulated transcription factor with ligand activated mechanism. This protein has been characterized as the regulator of responses to xenobiotic substances within a biological organism. AHR has a high affinity to TCDD and it is also called the dioxin receptor for this feature. For example it is known to disturb maturation of offspring in mice when there is prenatal exposure to TCDD(Takeda et al. 2014). AHR has earlier been seen only as a xenobiotic response unit but other roles have also been suggested such as a mediator in normal physiology and developmental physiology.(Furness & Whelan 2009, Herlin 2013) Developmental defects can be permanent and for this feature alone TCDD exposure during development is a subject worth looking at.

An inactive AHR is located in the cytoplasm. The ligands that can bind to it has to be lipophilic enough to penetrate the plasma membrane of the cell or have other ways of entering the cytoplasm . Once AHR is activated through ligand activation, it goes through a conformational change and translocates to nucleus. In the nucleus it exchanges its chaperone with aryl hydrocarbon nuclear translocator creating a new compound that is capable of initiating gene transcription. (Furness & Whelan 2009)

Different species and strains respond differently to TCDD most likely because of the different structure of AHR. Two rat strains (Long-Evans and Han/Wistar) for example show different magnitudes of bone changes with the Long-Evans strain being much more sensitive to TCDD.(Herlin 2010, Jämsä 2001)

1.4 Aim of the study

Aim of this study is to find out how early postnatal rat bone development is affected with different doses of TCDD in utero and a short seven day period of lactational exposure. Also gender differences are investigated.

2 Materials and methods

2.1 Animal Studies

Pregnant Sprague-Dawley rats were exposed to TCDD with five different doses on their 11th gestational day. After birth, 75 rat pups (38F, 37M) were divided to five groups depending on the dams dose and sacrificed on postnatal day (PND) 7. Doses were given as a single bodyweight(bw) -adjusted oral dose mixed with corn oil. Control group: 0µg/kg bw, Group 1: 0,03µg/kg bw, Group 2: 0,1µg/kg bw, Group 3: 0,3µg/kg bw, Group 4: 1,0µg/kg bw. Amount of pups and the size of each group can be found in Table 1. Some of the samples were excluded from the imaging because the glass containers were broken and the bones had dried or the bones were damaged during sample preparation.

Table 1. Doses, genders and group sizes

Dose	♂ **femurs**	♀**femurs**	♂**tibias**	♀**tibias**
0 µg/kg bw	7	8	7	8
0,03 µg/kg bw	8	7	8	7
0,1 µg/kg bw	7	8	6	8
0,3 µg/kg bw	7	7	7	7
1 µg/kg bw	8	5	8	5
Sum	**37**	**35**	**36**	**35**

2.2 Micro-computed tomography imaging

The tibias and femurs were imaged with a Skyscan 1174 compact µ-CT (Bruker microCT, Kontich, Belgium). Prior to imaging formalin fixed bone samples were stored in small glass bottles filled with 70% Ethanol. The bones were gently removed from the glass containers and wrapped in paper sheets wetted with 70% ethanol. Drying of the samples was to be avoided because it can affect the properties of the bone and cause shrinking. The bones were then inserted into an airtight capsule and imaged. The imaging settings were the same through-out the whole dataset and can be found in Table 2.

Table 2. Imaging settings

Setting	Value
Scanner	SkyScan1174v2
Partial Width	OFF
Camera binning	1x1
Source Voltage (kV)	50
Source Current (μA)	800
Image Pixel Size (μm)	6.73
Image Format	TIFF
Depth (bits)	16
Exposure (ms)	4000
Rotation Step (deg)	0.5
360 Rotation	YES
Flatfield Update	YES
Flatfield Correction	YES
Frame Averaging	ON (2)
Sharpening (%)	60
Random Movement	OFF
Geometrical Correction	ON

2.3 Reconstruction

The reconstruction settings for the acquired 2D images were kept the same through-out the whole dataset, thus making them comparable with each other. Skyscans own reconstruction software nRecon with GPUReconServer (Bruker microCT, Kontich, Belgium) was used for the reconstruction. Reconstruction settings can be found in Table 3. Post alignment value was determined manually for each sample with the fine tuning option.

Table 3. Reconstruction settings

Setting	Value
Post Alignment	Fine tuning
Pixel Size (µm)	6.73
Reconstruction Angular Range (deg)	360
Use 180+	OFF
Angular Step (deg)	0.5
Smoothing	2
Smoothing Kernel (Gaussian)	2
Ring Artifact Correction	10
Draw Scales	OFF
Object Bigger Than FOV	OFF
Undersampling Factor	1
Defect Pixel Mask (%)	25
Beam Hardening Correction (%)	40
Image Dynamic Range	0.0000-0.1200
Result File Type	BMP

2.4. Image orientation

The images were oriented using SkyScan Dataviewer version 1.4.4.(Bruker microCT, Kontich, Belgium) Length of the bones was also measured with Dataviewer. Volumes of interests (VOIs) were chosen from the full dataset where X- and Y-dimensions for the cross-sectional images were 550 pixels. Tibias and femurs were oriented so that the distal end of the bone is at the start of the image stack, the proximal end is at the end and the anterior side faces upwards in the cross-sectional images.

2.5. Image analysis

Images were analyzed with Skyscan CT-analyzer (CTAn1.14.4.1, Bruker microCT, Kontich, Belgium). The length of the bone acted as a reference for the region of interests (ROIs). 25% from the proximal end of the bone and 30% from the distal end of the bone were excluded from the analysis which left 45% of the bone (cortical part) for the analysis. The algorithm sequence can be found in Table 4.

Table 4. CTAn algorithms, two protocols(morphology and overall geometry)

Cortical analyzer (morphology)		**Cortical and medullar analyzer (geometry)**	
Name	Description	**Name**	Description
FIltering	Unsharp mask, 3D Space, Radius: 3, Amount: 50, Threshold: 60	**Tresholding**	Global, Low: 55, High:255
Tresholding	Adaptive: Mean of min and max values, 3D Space, Low: 55, High: 255, Radius: 2, Constant: 0	**Despeckle**	3D Space, Remove white speckles less than 10 voxels from image
Despeckle	3D Space, Remove white speckles less than 10 voxels from image	**Morphological operations**	3D Space, Type: Dilatation, Kernel: Round, Radius: 3, Apply to image
Despeckle	3D Space, Remove black speckles less than 10 voxels from image	**ROI shrink-wrap**	2D Space, Mode: Shrink-wrap, Stretch over holes: YES, Diameter: 30 pixels
Morphological operations	3D Space, Type: Closing, Kernel: Round, Radius: 1	**Reload**	Apply to image
ROI shrink-wrap	3D Space, Mode: Shrink-wrap, Stretch over holes: YES, Diameter: 40 voxels	**FIltering**	Unsharp mask, 3D Space, Radius: 3, Amount: 50, Treshold: 60
Morphological operations	3D Space, Type: Closing, Kernel: Round, Radius: 3, Apply to region of interest	**Tresholding**	Adaptive: Mean of min and max values, 3D Space, Pre-tresholding, Low: 55, High: 255, Radius: 2, Constant: 0
Save bitmaps	Apply to: Image inside ROI, File format: BMP	**Despeckle**	3D Space, Remove white speckles less than 10 voxels from image
Save bitmaps	Apply to: ROI, File Format: BMP	**Despeckle**	3D Space, Remove black speckles less than 10 voxels from image
2D analysis	All results	**Morphological operations**	3D Space, Type: Closing, Kernel: Round, Radius: 3, Apply to region of interest
3D analysis	Basic values: ALL, Additional values: ALL	**Despeckle**	3D Space, Type: Sweep all except the largest object
Reload	Apply to image	**Morphological operations**	3D Space, Type: Closing, Kernel: Round, Radius: 1
Histogram	3D Space, Unit: Grayscale indexes, Inside VOI: YES	**2D analysis**	All results
		3D analysis	Basic values: ALL, Additional values: ALL
		Save bitmaps	Image inside ROI, ROI

Bone mineral density (BMD) was also calculated using two calcium hydroxyapatite phantoms with known densities of 250 and 750 mg/cm3. BMD values were calculated as one mean value of the cortical part. CTAn parameters used to test differences between groups and control group can be found in Table 5 with their abbreviations also. Used abbreviations follow the guidelines for assessment of bone microstructure in rodents using μCT (Bouxsein et al. 2010).

Table 5. CTAn parameters

2D parameters with medullar area	**3D parameters with medullar area**	**3D parameters without medullar area**
Mean cross-sectional bone area(**M.Ct.Ar**)	Bone Volume(**BV**)	Bone volume/Tissue volume=Bone volume fraction(**BV/TV**)
Mean medullar area(**M.Ma.Ar**)	Volume of pore space(**Po.V**)	Bone surface/Tissue volume=Bone surface density(**BS/TV**)
Mean cross-sectional medullar perimeter(**Ec.Pm**)		Bone surface/Bone volume=Specific bone surface(**BS/BV**)
Mean cross-sectional bone perimeter(**Ps.Pm**)		Connectivity density(**Conn.D**)
cross-sectional thickness(**Ct.Th**)		Structural model index(**SMI**)
Mean polar moment of inertia(**J**)		Trabecular number(**Tb.N**)
Mean eccentricity(**M.E**)		Trabecular thickness(**Tb.Th**)
		Trabecular separation(**Tb.Sp**)
		Degree of anisotropy(**DA**)

2.6 Statistical analysis

Statistical analyses were done with IBM SPSS 20.0. Analysis of variance and post hoc Dunnets test was used to compare means between control and groups 1-4. Male bones and female bones were analyzed separately. Reported values are mean pixel values from CTAn. Group differences were considered as statistically significant when p-values were lower than 0.05.

3 Results

3.1 Male femurs

BV/TV was decreased 11.0% in 0.1 µg/kg (p=0.003) but no changes in BV/TV was seen in other groups. BS/TV was increased 6.5% and 6.2% with 0.1µg/kg (p=0.12) and 0.3 µg/kg (p=0.019), respectively but no significant change was seen with 0.03µg/kg and 1µg/kg. Conn.D was increased 19.4% with the dose 0.3 µg/kg (p=0.031) but no changes was seen in other groups. All other test parameters showed no statistically significant changes. Boxplots of male femur parameters can be seen in Figure 1

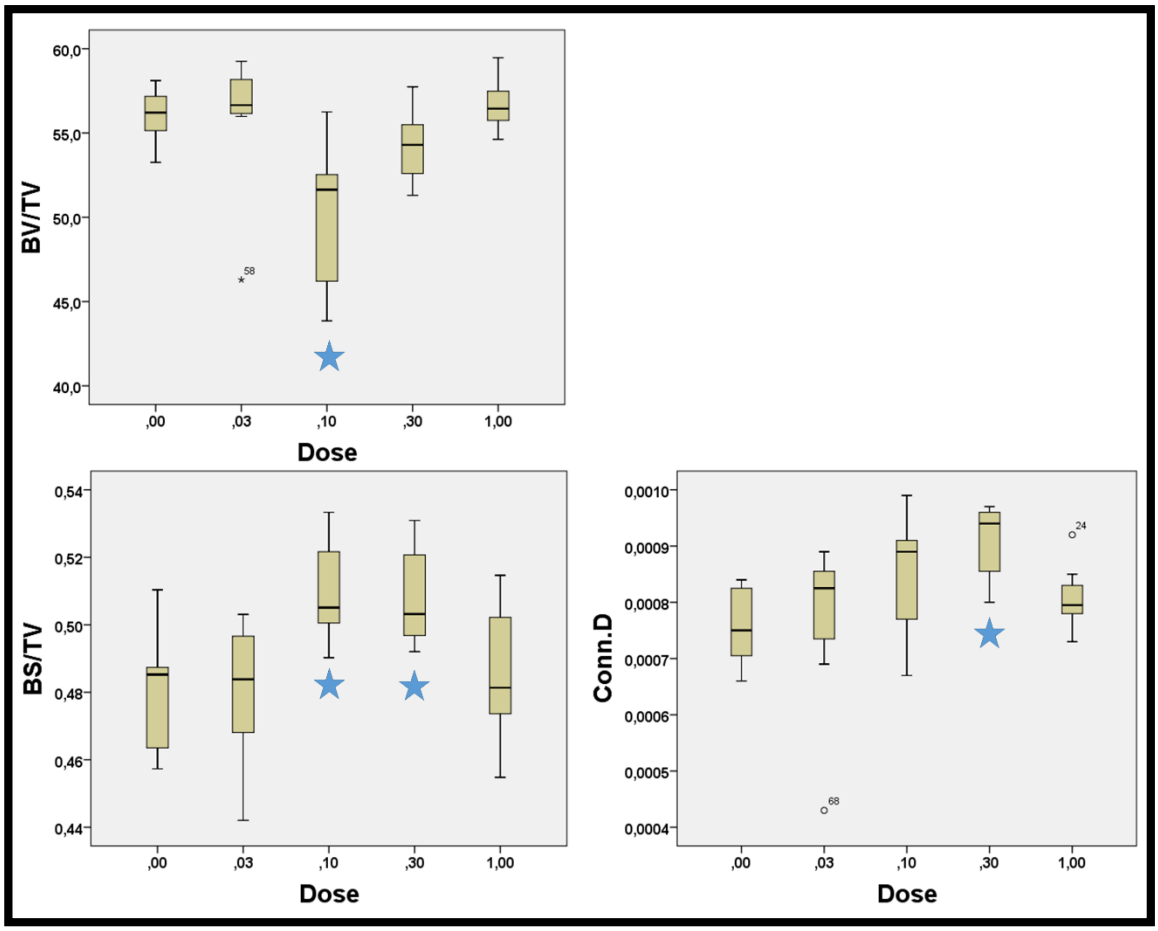

Figure 1. Boxplots of femoral structure in males. Statistical significance indicated with a blue star.

3.2 Male tibias

M.E was decreased by 10.5% and 9.3 with 0.03µg/kg (p=0.003) and 0.30µg/kg (p=0.010) respectively but not with other doses. M.Ma.Ar was decreased by 17.5% with only the highest dose (p=0.010).Ps.Pm was decreased by 7.8% with the highest dose (p=0.013) but not with the lower doses. Ec.Pm was decreased by 7.2% with only the highest dose(p=0.044) Tb.Th was decreased by 6.45% and 6.5% with doses 0.1µg/kg (p=0.001) and 0.3 µg/kg (p=0.0001). Tb.Sp was decreased by 16.0% and 14.6% with doses 0.1µg/kg (p=0.012) and 0.3µg/kg (p=0.017) respectively. Tb.N increased by 6.0% and 5.2% with doses 0.1µg/kg (p=0.001) and 0.3µg/kg (p=0.003) respectively. Boxplots of male tibia parameters can be seen in Figure 2 and Figure 3.

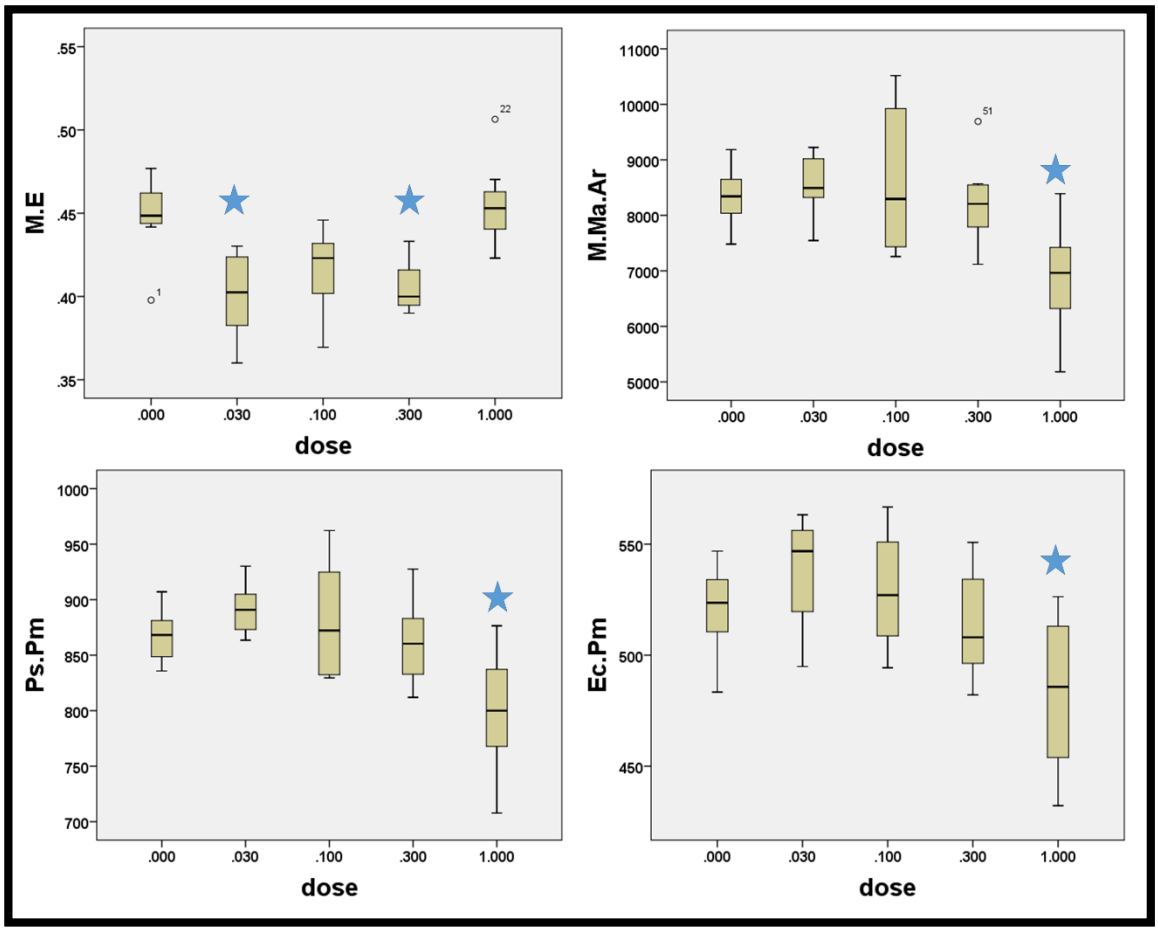

Figure 2. Boxplots of tibial geometry parameters in males. Statistical significance indicated with a blue star.

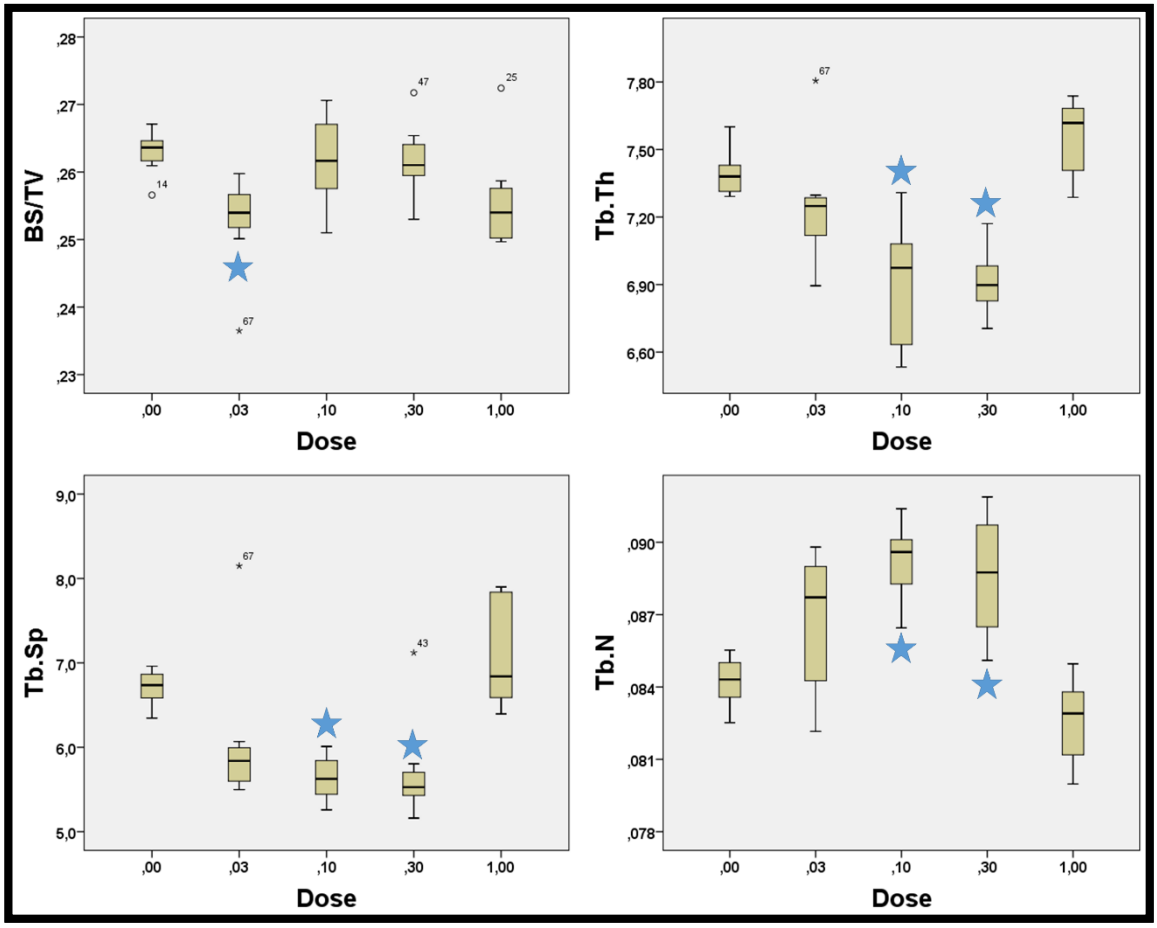

Figure 3 Boxplots of tibial structure in males. Statistical significance indicated with blue star.

3.3 Female femurs

M.Ma.Ar was decresead by 14.8% only in the 0.1µg/kg group

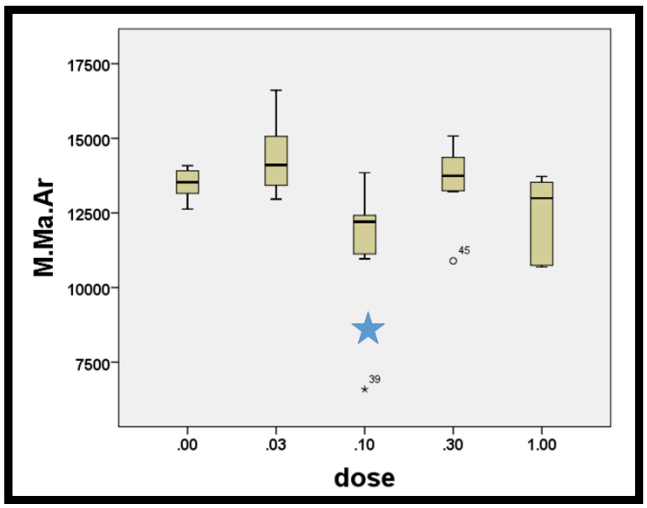

Figure 4 Boxplots of femoral geometry parameters in females. Statistical significance indicated with a blue star.

3.4 Female tibias

Tb.Th was decreased by 5.6-7.9% in all groups except the highest dose ($p<0.003$). Tb.Sp was decreased by 13.4-17.1% in all groups except the highest dose ($p<0.003$). Tb.N was increased by 5.2-7.8% in all groups except the highest dose ($p<0.003$). Boxplots of female tibia parameters can be seen in Figure 4.

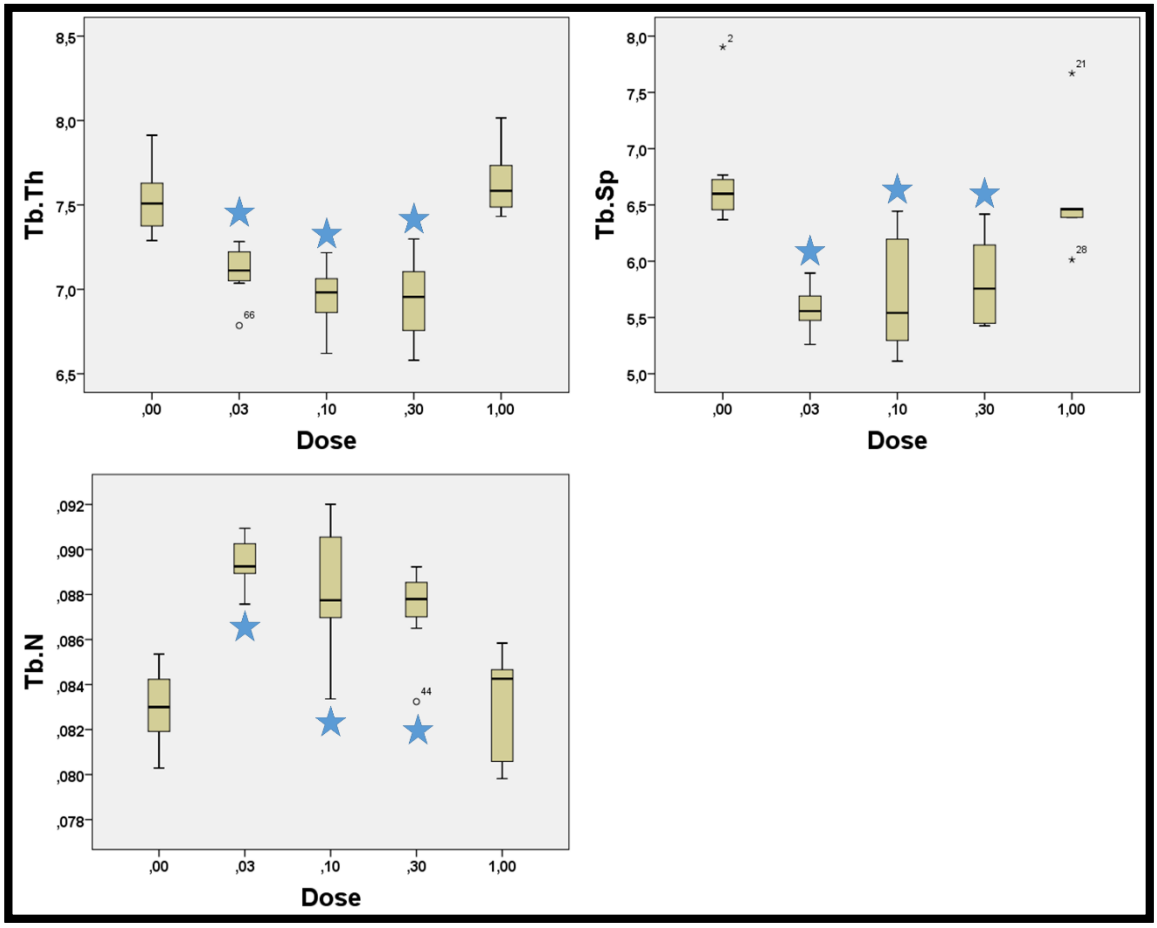

Figure 5. Boxplots of tibial structure in females. Statistical significance indicated with a blue star.

3.5 Other results

Also no statistically significant differences were found in bone length or BMD between any groups

Visual representations of cross-sectional binarized images from all groups can be seen in figures 6 to 9

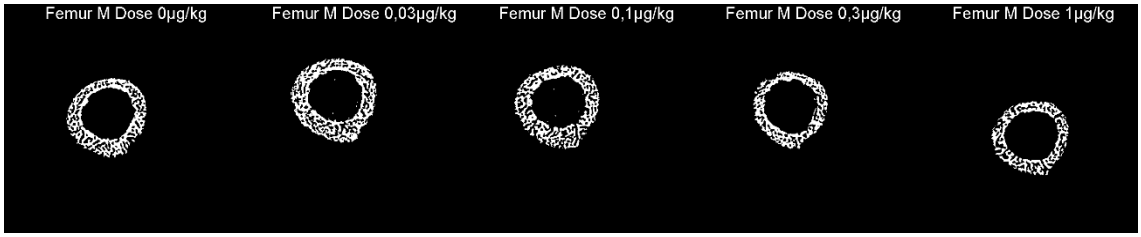

Figure 6. Cross-sectional binarized images from the middle of the ROI. Male femurs chosen randomly between test subjects

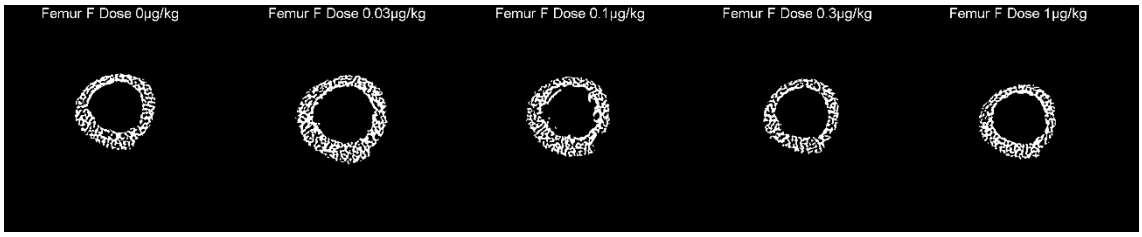

Figure 7 Cross-sectional binarized images from the middle of the ROI. Female femurs chosen randomly between test subjects

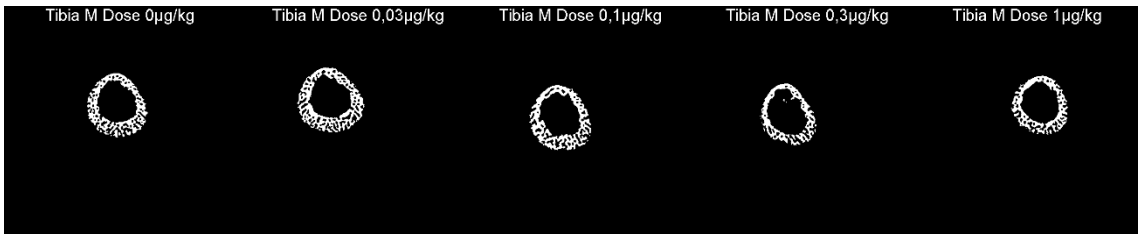

Figure 8. Cross-sectional binarized images from the middle of the ROI. Male tibias chosen randomly between test subjects

Figure 9. Cross-sectional binarized images from the middle of the ROI. Female tibias chosen randomly between test subjects

4 Discussion

In this study, the aim was to find out if combined *in utero* and lactational TCDD changes bone morphology in one week old rat pups. The rat legs were studied in both genders. Gender differences in acute toxicity of dioxins in mice, rats, monkeys and many other animals have been reported (Hermsen et al. 2008, Pohjanvirta & Tuomisto 1994, Pohjanvirta et al. 2012), such as the difference in

TCDD-sensitive rat strains where females are approximately half as resistant to a lethal dose whereas in different substrains of mice the case is opposite as females are the more resistant gender and can be over 10 fold more resistant (Pohjanvirta et al. 2012). However, the acute lethality varies greatly among species and for example the lethal dose of TCDD for a guinea pig is 1µg/kg while in hamster it is 5100µg/kg(Pohjanvirta & Tuomisto 1994).

There were gender differences also in this study. Samples from male rats had significant changes in bone morphology in both femurs and tibias and significant changes in geometry parameters in tibias, while female samples had significant morphological changes in tibias and only 0.1µg/kg group had a decrease in femoral medullar area. This contrasts to the previous results about rat gender sensitivity to TCDD(Pohjanvirta et al. 2012). Based on the results, it is hard to draw any conclusions on gender differences in this study, because of the small sample set and high variance within groups.

As immature cortical bone is woven (porous) trabecular 3D analysis is suitable for analyzing its structure. Both male and female tibias showed similar trends in the 3D parameters Tb.Th, Tb.Sp and Tb.N (Figures 3 and 4), where a decrease was seen in Tb.Th and Tb.Sp especially with the lowest doses and an increase in Tb.N in the low doses also. As both Tb.Th and Tb.Sp decrease, with simultaneous increase of Tb.N the bone morphology changes from a sponge-like structure to a sponge with more densely distributed thinner trabeculae. These results could also explain why changes in BV/TV are not found in tibias because the concurrent rise in Tb.N in contrast to Tb.Th and Tb.Sp negates the the change in bone and tissue volume ratio. These changes could indicate that the structural maturation has not been affected but the rate of maturation has decreased

Decrease in bone mineralization, cross-sectional geometry, bone length and bending strength were reported in a similar study where female rats were sacrificed on PND 35 or 70 with one gestational dose of TCDD (1µg/kg).(Finnilä et al. 2010) In this study, changes in the highest dosage were only seen in male tibias with a decrease in M.Ct.Ar and M.Ma.Ar which reflects the changes in cross-sectional geometry in figure 2. The differences in time of sacrifice (PND 7

vs. 35 and 70) might explain the differences between these two studies from two point of views: 1) lactational period (which in most rat and mice strains is 21 days(Sengupta 2013) is complete for the latter two. This means that a 3-fold exposure to TCDD is achieved from lactation. 2) Bones at PND 7 are still at the early phases of in transferring from woven to lamellar bone while bones at PND 35 and 70 are more advanced in maturation. Different lactational exposure would need more studying. Example setting of a study: groups with gestational exposure without lactational exposure and vice versa to see if lactational exposure or gestational exposure is the more aggressive pathway of exposure to TCDD and if different responses can be seen in the bone. This has been investigated earlier with male rats where their reproductive system was under observation. Both lactational and In utero exposure affected the offspring while certain responses were only associated with lactational exposure whereas other responses were only seen with in utero exposure.(Bjerke & Peterson 1994)

Male tibias had decreases in M.E, M.Ma.Ar, Ps.Pm and Ec.Pm. The geometry of the bone affects its mechanical properities. As M.E decreases, the mean shape of the cortical part approaches the shape of a circle. BS/TV increased in male femurs with the two intermediary doses which means that the cortical bone surface is more porous. Decreases with highest dose in medullar area, bone perimeter and medullar perimeter indicate a smaller bone size, which also affects the mechanical properties of the bone. In figure 8 are visual representations of cross-sectional slices but these changes are hard to see with the naked eye.

5 Conclusions

In conclusion, there are more severe morphological changes with the low doses (0.03-0.3µg/kg) than there is with the high dose (1µg/kg) in both female and male tibias. However, males seemed to be more sensitive as geometrical changes were also found in male tibias with the highest dose. Changes in bone morphology and geometry, can indicate a disturbed growth rate of bone which both can cause harm later in life.

6 References

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