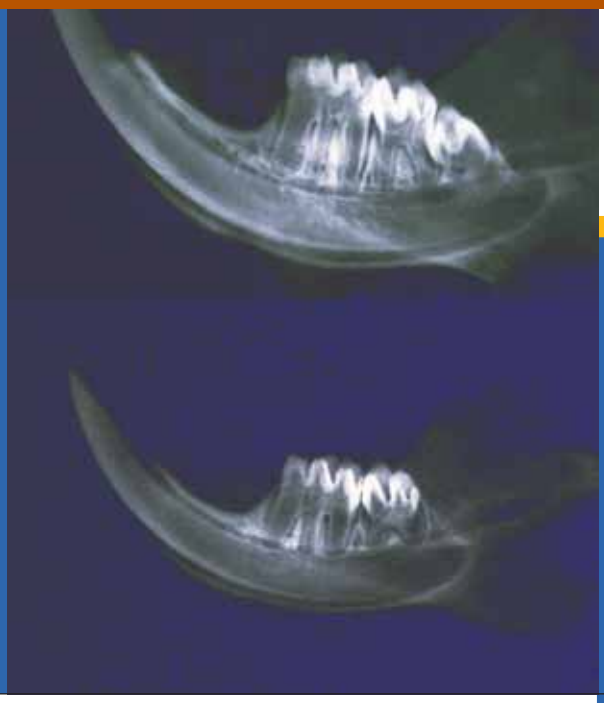

Hanna Miettinen

The Effects of TCDD on the Development of Teeth and Cortical Bone in Rats: Implications for Risk Assessment

Publications of the National Public Health Institute A 10/2006

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National Public Health Institute, Kuopio, Finland
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Department of Pharmacology and Toxicology,
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Kuopio 2006

Hanna Miettinen

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A C A D E M I C D I S S E R T A T I O N

To be presented with the permission of the Faculty of Pharmacy, University of Kuopio, for public examination in Auditorium ML2, Medistudia building, University of Kuopio, on October 6th, 2006 at 12 o'clock noon

Department of Environmental Health, National Public Health Institute, Kuopio, Finland

and

Department of Pharmacology and Toxicology, University of Kuopio, Finland

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is certainly one the most extensively studied compounds in the world. The huge interest in this compound is explained by the fact that it is the most potent man-made chemical, and it is often used as a model compound for a group of chemicals (polychlorinated dibenzo-*p*-dioxins and furans, (PCDD/Fs) also known as "dioxins" and polychlorinated biphenyls (PCBs)) that share similar physicochemical and toxicological properties, though with lesser degrees of potency. Dioxins have spread all over the face of the earth and, due to their stability and lipophilicity, they have accumulated in food chains.

Humans are exposed to PCDD/Fs and PCBs continuously *via* the diet; and in Scandinavia, the main source is fatty Baltic fish. European Commission has given Finland and Sweden a temporary exemption to use Baltic fish, and this matter will be revisited again in 2006.

TCDD is able to elicit a wide variety of harmful effects in laboratory animals. These effects are mainly mediated *via* the aryl hydrocarbon receptor (AHR). Even though cancer has traditionally been used in the risk assessment, new data have revealed that developmental defects, such as alterations in reproductive organ development, are more sensitive to TCDD than cancer and of great importance.

The present study describes new developmental defects induced by TCDD: alterations in tooth and bone development. Using three differentially sensitive rat lines it was demonstrated that the development of rodent dentition is disturbed at very low doses that are relevant for risk assessment, and that there are no major differences in sensitivity among lines. Changes in cortical bone development were also found, but only in the most sensitive rat line and only at the highest maternal dose level.

As molar tooth cannot be remodeled, developmental defects in dentition are permanent. Previous studies have shown that also humans respond to PCDD/F exposure with altered dental development. Due to the extreme sensitivity of dentition to TCDD, alterations in dental development, such as deviations in enamel structure in humans, could be considered as a sensitive, though not necessarily an exclusive, indicator for dioxin exposure during early life.

Keywords: TCDD, PCDD/Fs, tooth, bone, development

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TIIVISTELMÄ

2,3,7,8-Tetraklooridibentso-*p*-dioksiini (TCDD) on kemikaali, jota on tutkittu varmaankin enemmän kuin mitään toista kemikaalia. Tätä valtavaa kiinnostusta selittää se, että TCDD aiheuttaa haittavaikutuksia äärimmäisen pienillä annoksilla, ja sitä voidaan käyttää dibentso-*p*-dioksiinien ja -furaanien (PCDD/F) sekä polykloorattujen bifenyyleiden (PCB) malliaineena. Näillä aineilla on samankaltaiset molekyylirakenteet ja haittavaikutukset, mutta annokset, jotka aiheuttavat näitä haittoja, ovat eri johdoksilla hyvin erilaiset. Dioksiinit ovat levinneet kaikkialle maapallolla, ja koska ne ovat kestäviä ja rasvaliukoisia, ne kertyvät ravintoketjuissa.

Ihmiset altistuvat PCDD/F- ja PCB-yhdisteille pääasiassa ravinnon kautta. Skandinaviassa suurin altistus tulee rasvaisista Itämeren kaloista. Euroopan komissio on antanut Suomelle ja Ruotsille väliaikaisen poikkeusluvan käyttää Itämeren kalaa, luvan perusteita arvioidaan uudelleen vuonna 2006.

TCDD aiheuttaa laajan kirjon haittavaikutuksia koe-eläimissä. Nämä vaikutukset välittyvät pääasiassa aryylihiilivetyreseptorin (AHR) kautta. Vaikka riskinarvioissa on pääasiassa käytetty syöpää tärkeimpänä haittavaikutuksena, uudet kokeet osoittavat, että kehityshäiriöt, kuten muutokset sukupuolielinten kehityksessä, ovat erittäin herkkiä ja tärkeitä dioksiinien riskinarviossa.

Väitöskirjatutkimuksessani kuvataan kaksi uutta kehityshäiriötä, jota TCDD aiheuttaa: muutokset hampaiston ja kuoriluun kehityksessä. Kolmea herkkyydeltään erilaista rottalinjaa käyttäen osoitimme että rotan hampaiston kehitys häiriintyy hyvin alhaisilla annostasoilla, ja että nämä annokset ovat relevantteja riskinarvioinnin kannalta. Hampaiden kehitys häiriintyy riippumatta yleisestä TCDD-herkkyydestä. Tutkimuksessa huomasimme myös muutoksia kuoriluun kehityksessä, mutta vain korkeimmalla annostasolla ja vain herkimmässä rottalinjassa.

Koska hampaisto ei uusiudu kehityttyään, sen kehityshäiriöt ovat pysyviä. Aiemmat tutkimukset ovat osoittaneet, että PCDD/F-altistus muuttaa hampaiston kehitystä myös ihmisellä. Koska rotan hampaiden kehitys on erittäin herkkä TCDD:lle, muutoksia hampaan kehityksessä, kuten ihmisillä kiilteen rakennehäiriöitä voi ehdottaa herkäksi bioindikaattoriksi aikaiselle PCDD/F-altistukselle, tosin myös muut aineet saattavat häiritä kiilteen kehitystä.

Avainsanat: TCDD, PCDD/F, hampaat, luu, kehitys

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ABBREVIATIONS AND DEFINITIONS

AHR	Aryl hydrocarbon receptor
Ahr	The gene coding for AHR
$Ahr^{hw/hw}$	Allele of *Ahr* locus; found in TCDD-resistant Han/Wistar rats and in line A rats
ARNT	AH receptor nuclear translocator (it does not in fact translocate AHR): it dimerizes with the AHR, after which the complex binds to DNA
$B^{hw/hw}$	Allele of unknown *B* gene; found in TCDD-resistant Han/Wistar rats and in line B rats
BMD	Cortical bone mineral density of a tubular bone
BSE	Back scatter electrons
CA-AHR	Constitutively active AHR
CSA	Cross-sectional area of cortex (CSA) of a tubular bone
CYP1A2	Cytochrome P450 1A2, is a member of the cytochrome P450 mixed-function oxidase system, and is involved in the metabolism of xenobiotics in the body.
DDE	Dichlorodiphenyldichloroethylene
Dioxin	Inconsistently used term: in the literature may refer to TCDD alone, PCDD/Fs as a group or both PCDD/Fs and certain PCBs. In this thesis, dioxins refer to PCDD/Fs.
DL-PCBs	Dioxin-like PCBs: PCB congeners that can induce similar toxic effects as dioxins. DL-PCBs have a maximum of one chlorine atom in the *ortho*-position to the carbon-carbon bond between the two benzene rings.
EDS	Energy dispersive spectrometry
ENDO	Endosteal circumference of a tubular bone
GD	Gestation day. GD0 is the day when spermatozoa are found in vaginal smear or a vaginal plug is found
H/W	Han/Wistar (*Kuopio*) rat strain

LD50	Lethal dose 50%: the dose that is estimated to kill half of the animals in an acute toxicity test
L-E	Long-Evans (*Turku*/AB) rat strain
Line A	A rat line originated from H/W and L-E rats; assumed to have the genotype $Ahr^{hw/hw}B^{wt/wt}$; very resistant to TCDD
Line B	A rat line originated from H/W and L-E rats; assumed to have the genotype $Ahr^{wt/wt}B^{hw/hw}$; moderately resistant to TCDD
Line C	A rat line originated from H/W and L-E rats; assumed to have the genotype $Ahr^{wt/wt}B^{wt/wt}$; sensitive to TCDD
LOAEL	Lowest observed adverse effect level
MDL	Mesio-distal length of molar
NOAEL	No observed adverse effect level
PCBs	Polychlorinated biphenyls
PCDD/Fs	Polychlorinated dibenzo-*p*-dioxins and dibenzofurans, also known as “dioxins”
PERI	Periosteal circumference of a tubular bone
PMI	Polar cross-sectional moment of inertia
PND	Postnatal day; days after birth. The day when delivery is detected is PND0.
pQCT	Peripheral quantitative computer tomography
s.c.	Subcutaneous. Exposure that is given beneath skin.
SEM	Scanning electron microscopy
TBBMC	total body bone mineral content
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin: the most potent congener of dioxins
TDI	Tolerable daily intake: the amount of a contaminant that is safe for humans to take in every day for an entire lifetime
TEF	Toxic equivalence factor: potency of a compound to induce dioxin-like effects in relation to TCDD with TEF value 1

TEQ	Toxic equivalence quantity: sum function of toxicity of all congeners: amount of a congener multiplied by the TEF value of the congener
WT	Wild type mice. Mice with normal, not-modified genotype that serves as control for genetically modified mice line.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I **Kattainen H.**, Tuukkanen J., Simanainen U., Tuomisto J. T., Kovero O., Lukinmaa P. L., Alaluusua S., Tuomisto J., Viluksela M. (2001) In utero/lactational 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure impairs molar tooth development in rats. Toxicology and Applied Pharmacology. 174:216-224.

II **Miettinen H.M.**, Alaluusua S., Tuomisto J., Viluksela M. (2002) Effect of TCDD on rat molar development: the critical time window. Toxicology and Applied Pharmacology. 184:57-66.

III **Miettinen H.M.,** Pulkkinen P., Jämsä T., Koistinen J., Simanainen U., Tuomisto J., Tuukkanen J., Viluksela M. (2005) Effects of *in utero* and lactational TCDD exposure on bone development in differentially sensitive rat lines. Toxicological Sciences. 85:1003-1012.

IV **Miettinen H.M.,** Sorvari R., Alaluusua S., Murtomaa M., Tuukkanen J., Viluksela M. (2006) The effect of perinatal TCDD exposure on caries susceptibility in rats. Toxicological Sciences. 91:568-575.

1 INTRODUCTION

The term “dioxins" gathers together a group of notorious chemicals[1] sharing similar structural (Fig 1) and physical and biological properties. These compounds are both environmentally and metabolically stable and due to their lipophilicity, they tend to accumulate in food chains. Each congener has its own individual potency to induce toxic effects; the most toxic dioxin is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Polychlorinated dibenzo-p-dioxins and –furans (PCDD/Fs) have never been intentionally produced except for research purposes, but instead they tend to occur as impurities in polychlorinated biphenyls (PCBs) and analogous chemicals. PCDD/Fs are formed in metal industries and in burning processes, especially in waste incineration (Pohjanvirta and Tuomisto, 1994). Dioxins have spread around the world, mainly due to their formation in combustion processes in the presence of chlorine and with metal catalysts and the use of dioxin-contaminated chemicals between the 1950’s and 1970’s. Since that time, their environmental levels have been declining. The main route of exposure for humans is diet, especially fatty milk and meat products. In Finland, Baltic herring represent the greatest contributor to dioxin exposure, since the Baltic Sea is heavily contaminated with dioxins (Kiviranta *et al.*, 2001; Charnley and Doull, 2005).

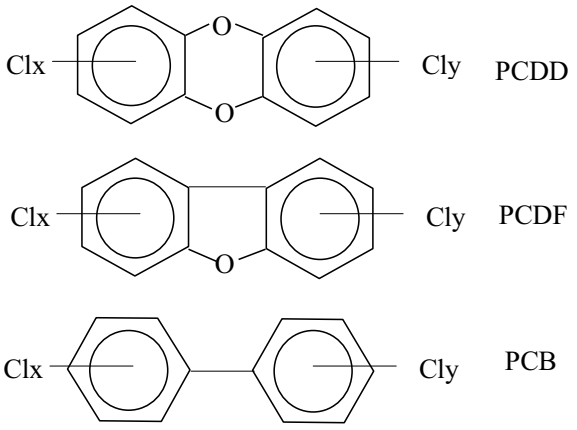

Figure 1. Chemical structure of PCDD/Fs and PCBs

[1] Polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzo-*p*-furans and polychlorinated biphenyls (PCBs). Note that there are both dioxin-like and non-dioxin-like PCBs.

The daily intake of PCDD/Fs and dioxin-like PCBs is presently estimated to be below 2 pg TEQ/kg (TEQ means the sum of the amounts of PCDD/Fs and dioxin-like PCBs multiplied by their relative toxic potency) in adult populations in Finland and USA (Kiviranta *et al.*, 2001; Charnley and Doull, 2005), which is in the range of the tolerable daily intake (1-4 pg/kg) according to the World Health Organisation (WHO, 2000). The case for breast-fed infants is very different. Their daily intake on body weight basis exceeds that of adults by one or two orders of magnitude (Pluim *et al.*, 1993), because during lactation, maternal fat tissue and the dioxins present in the body fat are mobilized and transferred to the infant *via* the milk.

TCDD, used widely in toxicological research as the model compound for dioxins, elicits many biological responses in laboratory animals. These effects range from mild clinicochemical changes to a lethal wasting syndrome, and include immunosuppression, thymus athropy, tumour promotion as well as teratogenic defects. Interestingly, there is a great inter- and intraspecies variation in the sensitivity to some but not to all of these effects. LD50 values for two of the rat strains used in our laboratory differ by 1000 fold (Pohjanvirta *et al.*, 1987; Tuomisto *et al.*, 1999), but typical developmental defects, hydronephrosis and cleft palate, occurred at similar dose levels in Han/Wistar and Long-Evans rats, respectively (Huuskonen *et al.*, 1994). The significance of developmental defects from the risk assessment point of view is emphasized by the fact that embryos and juvenile offspring are generally affected at low dose levels, and the defects may be irreversible. Very low doses of TCDD (0.05-1.0 μg/kg to rat dam) alter sexual behaviour in male offspring and induce morphological changes in the reproductive organs (Mably *et al.*, 1992a; Mably *et al.*, 1992b; Mably *et al.*, 1992c; Gray *et al.*, 1997a; Gray *et al.*, 1997b; Hamm *et al.*, 2000).

The present study focused on the TCDD-induced effects on developing tooth and cortical bone in rats. The tooth and bone effects caused by TCDD are intriguing, because they have been noted in many species of laboratory animals even though they have not been systematically studied (Hornung *et al.*, 1999; Allen and Leamy, 2001; Mattingly *et al.*, 2001; Bowers *et al.*, 2004; Yasuda et al., 2005a). Furthermore, bone lesions in wild animals have been associated with exposure to dioxin-like chemicals (Gilbertson *et al.*, 1991; Bergman *et al.*, 1992). Developmental tooth defects, such as missing permanent teeth, have been reported in humans accidentally exposed to TCDD and related compounds and even in a normal child population exposed via mothers' milk (Alaluusua *et al.*, 1996b; Hamada, 1996; Alaluusua *et al.*, 1999; Alaluusua *et al.*, 2004). The results of the present study provide further experimental information of these defects in rats and the results are applicable for risk assessment purposes.

2 REVIEW OF THE LITERATURE

2.1 Background

TCDD, the most potent dioxin congener, causes a broad spectrum of toxic responses. These include reproductive and developmental defects, immunotoxicity, thymus atrophy, chloracne, wasting syndrome, hepatotoxicity and cancer in experimental animals (Pohjanvirta and Tuomisto, 1994). Some of the toxic defects, e.g. thymus atrophy, hepatotoxicity and wasting syndrome, are induced only after treatment with a relatively high dose, whereas others, such as suppressed immune response and altered sexual development (Fig 2), are induced at very low doses. One peculiar feature of TCDD toxicity complicating the risk assessment is that there are enormous inter- and intraspecies sensitivity differences to some, but not to all, of the toxic endpoints. Therefore agencies responsible for risk assessment have defined very variable tolerable daily intakes (TDI): WHO recommends 1-4 pg/kg (WHO, 2000), whereas the TDI set by USEPA is 0.001-0.01 pg/kg. The basis for risk assessment has been either cancer (USEPA) or developmental defects (WHO). Recently risk assessment has focused on reproductive and developmental defects instead of cancer, because these effects are observed at lower exposure levels and in general TCDD affects embryos and juveniles at similar dose levels regardless of species. Moreover, most agencies do not consider linear extrapolation of cancer risk appropriate, because TCDD is not genotoxic but acts as a tumour promoter. Before the present experimental studies were initiated, a few studies had indicated that developing human teeth, especially mineralization in molars, could have been affected by dioxins after high accidental or even at background exposure (Fig 2) (Hamada, 1996; Alaluusua *et al.*, 1999). However, there were experimental studies performed only in adult rats that described somewhat conflicting results from that obtained in humans: TCDD caused pulpal perforation in continuously erupting rat incisors. The present study aimed in dissecting possible developmental tooth and bone defects in experimental rats at doses relevant for risk assessment, and establishing the biological plausibility of the human teeth defects.

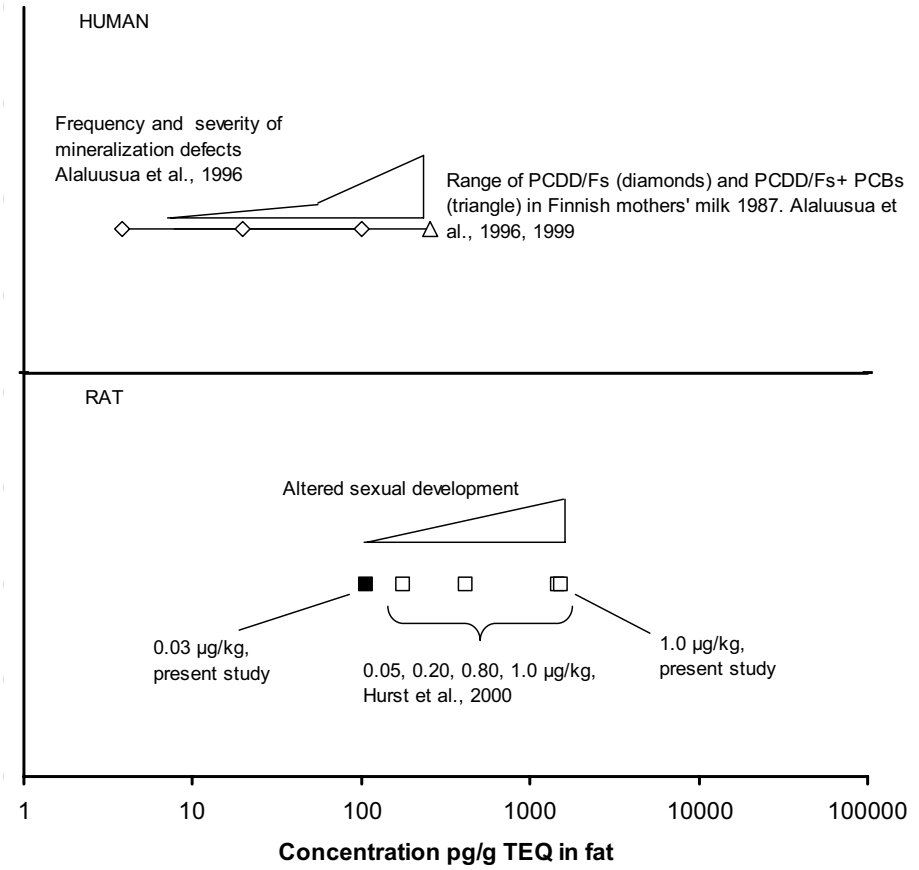

Figure 2. Maternal fat based PCDD/F concentrations and reported developmental defects in offspring. Open diamonds (minimum, mean and maximum of PCDD/Fs) and triangle (maximum of PCDD/Fs and DL-PCBs) denote measured concentrations in humans, open squares in rats. The lowest rat concentration (solid square) is a calculated value based on the data from Hurst *et al.*, 2000.

PCDD/Fs can cause a plethora of effects in laboratory animals and toxicity induced by PCDD/Fs has been reported in wild animals and in accidentally exposed humans. TCDD, the most potent congener of PCDD/Fs has been considered as a model compound for the entire group. TCDD affects multiple organs and functions, one hallmark sign being eventually lethal wasting syndrome. Most, but not all, of the effects have been detected in all studied

vertebrates (Birnbaum and Tuomisto, 2000). Even though sensitivity to lethality in adult animals may vary by up to 1000 fold among rat strains and laboratory animal species (Pohjanvirta *et al.*, 1987; Tuomisto *et al.*, 1999), embryos and developing offspring are affected at similar dose levels in strains and species. The classical teratological effects of TCDD in mice are hydronephrosis and cleft palate, the former being more sensitive endpoint than the latter (Couture *et al.*, 1990). TCDD also decreases foetal weight and foetal thymic weight, increases fetolethality and suppresses the immune function of offspring. More sensitive endpoints of developmental TCDD toxicity include alterations in reproductive organ weight and function (Mably *et al.*, 1992a; Mably *et al.*, 1992b; Mably *et al.*, 1992c; Gray *et al.*, 1997a; Gray *et al.*, 1997b; Hamm *et al.*, 2000).

TCDD is well absorbed well from gastrointestinal tract, which is the main route of exposure (Birnbaum and Tuomisto, 2000). After absorption, TCDD distributes mainly to adipose tissue but high doses induce CYP1A2 expression with consequent hepatic sequestration (Diliberto *et al.*, 1997; Birnbaum and Tuomisto, 2000). It is eliminated very slowly after background dietary exposure; its half-time in adults is between 5-10 years at these low exposure levels (Kreuzer *et al.*, 1997), whereas it can be as short as 2 years after high exposure (Michalek *et al.*, 2002). In newborn infants, the half-time is approximately 5 months (Kreuzer *et al.*, 1997). Even the developing embryo can be exposed to TCDD since the molecule is able to cross the placenta. In laboratory animals, TCDD has been measured in embryos 30 minutes after maternal p.o. dosing (Abbott *et al.*, 1996). Embryonic exposure is limited but detectable also in humans: in German still-born foetuses an average of 10 I-TEQ pg/g lipid of adipose tissue was detected in 1990-1991 (Kreuzer *et al.*, 1997 and personal communication). Lactational exposure on a body weight basis is high due to the fact that PCDD/Fs are mobilized from maternal adipose tissue during lactation.

2.2 The mechanism of dioxin toxicity

Most of the toxic effects of TCDD are mediated *via* the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor. AHR is a cytosolic receptor that belongs to the basic helix-loop-helix/PAS (bHLH/PAS) protein family. When a ligand binds to AHR, the complex translocates to the nucleus and chaperone proteins attached to the AHR dissociate. The ligand-receptor complex dimerizes with aryl hydrocarbon receptor nuclear translocator (ARNT), another member of the bHLH/PAS protein family. Subsequently the dimer binds to the DNA at a site called

the dioxin responsive element (DRE) where it activates gene expression. The best-known example of this cascade is the activation of *Cyp1a1*, and it is believed that the mechanism is similar for other genes (Whitlock, 1999). However, the specific mechanism of dioxin toxicity is still unclear in spite all theoretical interest and extensive research. The lack of functional *Ahr* prevents the most symptoms of TCDD toxicity (Fernandez-Salguero *et al.*, 1996; Lin *et al.*, 2001). Using a genetically modified animal model in which AHR could not transfer to nucleus and thus not bind to DRE, Bunger and co-workers proved that TCDD induced hepatomegaly, thymus athropy and cleft palate were mediated *via* AHR (Bunger *et al.*, 2003). A restricted expression of ARNT also attenuated TCDD induced thymic involution and hepatotoxicity (Walisser *et al.*, 2004). The classical teratological effects of TCDD, hydronephrosis and cleft palate are not be induced in mice embryos without functional AHR (Mimura *et al.*, 1997). Taken together these results stress the importance of the AHR/ARNT pathway in dioxin induced toxicity.

There may be alternative mechanisms of minor importance mediating TCDD-induced toxicity. In AHR knock out mice, TCDD-induced developmental effects were in some cases similar to, but in other cases opposite of those occurring in normal littermates (Lin *et al.*, 2001). Tuomisto *et al.* suggested that mortality in the TCDD-resistant H/W rat strain would be mediated in an AHR independent manner (Tuomisto *et al.*, 1999) and Peters *et al.* proposed that an AHR independent mechanism would contribute to the developmental toxicity associated with TCDD (Peters *et al.*, 1999). On the other hand, very few genes are up- or downregulated by TCDD independent of the AHR (Tijet *et al.*, 2006). Signalling via epidermal growth factor receptor (EGFR) is involved in TCDD-induced dental toxicity in embryonic mouse teeth exposed to TCDD *in vitro*. $EGFR^{-/-}$ teeth develop normally despite TCDD exposure, whereas in $EGFR^{+/+}$ and $EGFR^{+/-}$ embryos, TCDD prevented dentin matrix mineralization and enamel matrix deposition. Supplementing with EGF prevented most of the adverse effects of TCDD on the $EGFR^{+/+}$ teeth (Partanen *et al.*, 1998).

AHR, as a part of bHLH-PAS gene superfamily, regulates the expression of certain subtypes of cytochrome P450. In addition, it has physiological roles that have not been clarified yet. For example, little is known about its possible endogenous ligands; two possible physiological ligands are indirubin and indigo (Adachi *et al.*, 2001; Sugihara *et al.*, 2004). AHR homologs occur in multicellular organisms and during evolution, AHR gene has duplicated and diversified into at least three members of an AHR gene family: AHR1 (AHR), AHR2 and AHR repressor (AHRR). It appears that AHR homologs have regulative properties on some xenobiotic metabolizing enzymes only in vertebrates (Hahn, 2002). A large number of ligands bind to AHR. Theses include, in addition to PCDD/Fs, coplanar PCBs,

some polycyclic aromatic hydrocarbons, tryptophan metabolites and many flavone derivatives. Despite the structural divergence among ligands, the highest affinity ligands are hydrophobic, planar and of a defined size (Petrulis and Perdew, 2002). In adult rats, AHR is mainly expressed in the lung, thymus, kidney and liver, heart and spleen. During gestation, AHR is expressed in a spatially and temporally restricted manner in embryonic tissues, including neuroepithelium, heart, somites, liver, ectoderm, bone and muscle (Abbott *et al.*, 1995; Mattingly *et al.*, 2001; Sahlberg *et al.*, 2002). The wide expression does not mean that AHR expression is a prerequisite for viability. However, an AHR deficiency disrupts severely embryonic vascular development (Lahvis *et al.*, 2000), has a slight impact on cranial development (Peters *et al.*, 1999) and evokes a range of reproductive deficiencies in female mice (Pocar *et al.*, 2005).

TCDD affects AHR levels in a manner that depends on the dose, duration of exposure and the tissue or organism. In cell culture, AHR levels are rapidly depleted after exposure due to proteolytic degradation (Pollenz, 1996; Giannone *et al.*, 1998; Davarinos and Pollenz, 1999; Pollenz and Barbour, 2000). *In vivo*, TCDD exposure increases cytosolic AHR levels at low doses, but induces depletion of AHR at higher doses (Franc *et al.*, 2001a; Franc *et al.*, 2001b).

2.2.1 Description of the animal model

The three rat lines used in the present study originate from a very dioxin resistant H/W rat strain and a dioxin sensitive rat strain Long-Evans L-E. H/W rats have a mutated *Ahr* and another, still unidentified, gene that is associated with increased resistance to the lethality of dioxin. The genes were segregated by selective cross-breeding to create three new rat lines (Tuomisto *et al.*, 1999),. Line A rats have the mutated, H/W type $Ahr^{hw/hw}$, line B rats carry the resistance allele $B^{hw/hw}$ and line C rats have no resistance alleles. A point mutation in $Ahr^{hw/hw}$ results in loss of amino acids from the transactivation domain of the receptor protein and high resistance to some, but not all, endpoints of dioxin toxicity (Pohjanvirta *et al.*, 1998; Tuomisto *et al.*, 1999; Simanainen *et al.*, 2002). The unknown allele B^{hw} has a lesser influence on dioxin resistance (Tuomisto *et al.*, 1999; Simanainen *et al.*, 2003). The LD50 values against TCDD for line A, B, and C rats and for H/W and L-E rats are shown in Table 1.

Table 1. LD50 values and observed teratogenic effects for TCDD for line A, B and C rats and for H/W and L-E rats.

Rat line or strain	Presumed genotype	LD50 (μg/kg TCDD)		Teratogenesis at μg/kg [a)]
		Male	Female	
A	$Ahr^{hw/hw}$ $B^{wt/wt}$	>10 000	>2000	Not studied
B	$Ahr^{wt/wt}$ $B^{/hw/hw}$	830	410	Not studied
C	$Ahr^{wt/w\,t}B^{wt/wt}$	40	19	Not studied
H/W	$Ahr^{hw/hw}$ $B^{hw/hw}$	>10 000	>10 000	10; hydronephrosis
L-E	$Ahr^{wt/wt}$ $B^{wt/wt}$	18	7	5; cleft palate

a) Maternal dose (Huuskonen *et al.*, 1994)

The influence of Ahr^{hw} and B^{hw} on the dioxin resistance depends on the endpoint. The endpoints have been classified, based on short-term dose-response studies, into two categories (Tuomisto *et al.*, 1999; Simanainen *et al.*, 2002; Simanainen *et al.*, 2003). Type I endpoints are those for which dioxin sensitive and resistant rats show similar sensitivities. Examples of these endpoints are increased CYP1A1 activity and atrophy of thymus. For type II endpoints (e.g. weight loss and liver toxicity) the resistance alleles suppress the efficacy of TCDD, i.e. there are differences in the magnitude of the effect. Interestingly, even though type II endpoints are primarily high-dose effects, there are also some high-dose effects that belong to type I endpoints, such as incisor tooth defect in adult rats.

2.3 Teeth: structure and development

Teeth are found only among vertebrates. Teeth are useful for many purposes: mastication, fighting and communication of all forms. They can be either of uniform shape (homodont) as is found in most fish or show differences in morphology (heterodonty) (Thesleff and Nieminen, 2001). There are four groups of teeth in mammals: incisors, canines, premolars and molars. Dental shapes vary greatly among species due to different needs, the e.g. diet to be consumed.

There are three kinds of mineralized tissues in teeth: enamel, dentin and cementum. Cells unique to teeth, ameloblasts, odontoblasts and cementoblasts secrete the mineralized tissues. Teeth consist mainly of elements derived from the neural crest ectomesenchymal cells; the only epithelial component of tooth is enamel.

Enamel is acellular, insensitive and inert tissue covering the anatomical crown of teeth. It consists of about 96% inorganic material, mainly hydroxyapatite in crystalline form. Traces of organic material - remnants of proline-rich amelogenin and acidic glycosylated phosphoprotein secreted during organic matrix formation - envelope each crystallite. Since enamel is acellular, it cannot regenerate. On the other hand, ionic exchange can occur with the environment (Ten Cate, 1985).

Dentin forms the bulk of the tooth. It is hard, approximately 70% mineralized with hydroxyapatite crystals (Ten Cate, 1985). The organic component is mainly type I collagen (Thesleff, 1997), and it contains two unique proteins, i.e. dentin phosphoprotein and dentin sialoprotein that are not found in bone (Robey, 1996). There is no circulation in dentin and only cytoplasmic processes of the osteoblasts rather than cell bodies. However, odontoblasts embedded in the dentin are able to deposit more dentin should such a need arise. Dental pulp is nonmineralized connective tissue surrounded by dentin. Even if pulp is anatomically and functionally different from dentin, it has the same embryological origin as dentin. In addition, the actions of pulp – dentin formation, nutrition, protection and reparation – are related to dentin. Teeth only have nerves and blood vessels in the dental pulp entering from apical and accessory foramina (Ten Cate, 1985).

Acellular cementum covers the root of the teeth. It is a bonelike, avascular tissue consisting of organic matrix, mainly collagen and it is about 50% mineralized with hydroxyapatite crystals. Periodontal ligament fibres, anchored in the cementum and in the alveolar bone, attach the teeth to the surrounding alveolar bone (Ten Cate, 1985).

The development of teeth goes through successive stages named after the morphological features of each stage (Ten Cate, 1985; Thesleff, 1997). Initiation is first observable as a thickening of the stomodeal epithelium in the region of the future teeth. At the subsequent bud stage, represented as epithelial ingrowth into the underlining mesenchyme, the tooth germ is surrounded by condensed mesenchymal cells (Fig 3). When the tooth bud has reached its full size it folds and invaginates forming a cap-shaped structure. At the late bud stage and at the cap stage, the central cells of the dental epithelium form a cluster of condensed cells, the primary enamel knot. It is marked by the centralized expression of a number of molecules essential for normal tooth development. Enamel knot induction is a prerequisite for the tooth to develop from the bud stage to the cap stage and it has also been suggested that the primary enamel knot is an important regulator of tooth shape (Jernvall and Thesleff, 2000). During the cap stage, epithelial enamel (or dental) organ resembles a cap that surrounds mesenchymal dental papilla and it is flanked by mesenchymal dental follicle.

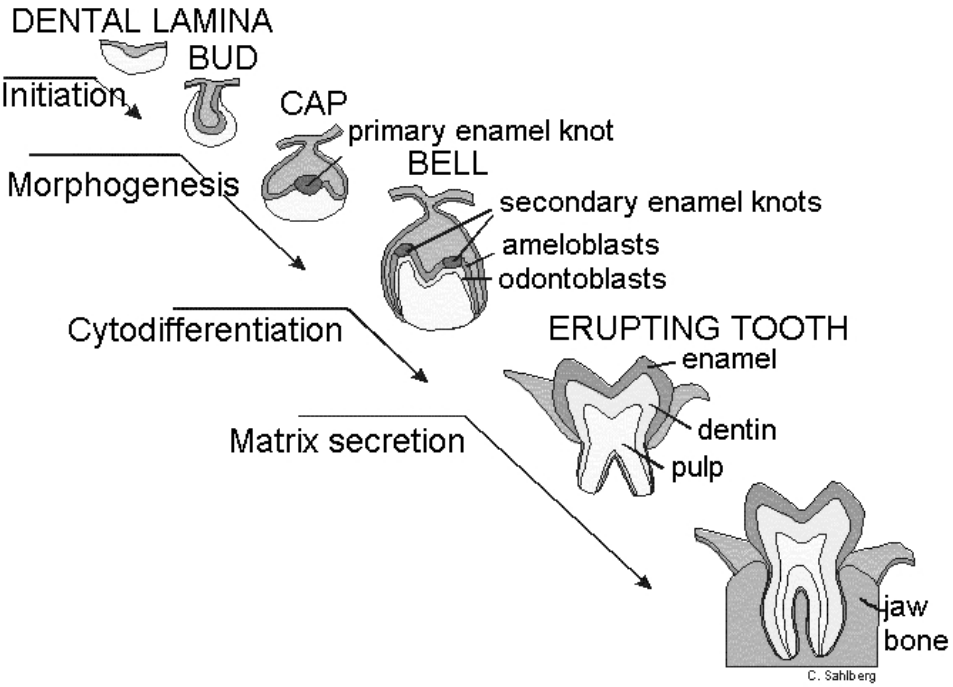

Figure 3. Development of a tooth (illustration courtesy of Carin Sahlberg).

Continuous growth of the tooth germ results in the shape of bell. The enamel knot is a transient structure that disappears apoptotically. After the apoptosis of the enamel knot in teeth with many cusps (like molars), new enamel knots called secondary enamel knots form at the locations of future cusp tips (Jernvall and Thesleff, 2000). Folding of the epithelium takes place in the same way as occurs with the primary enamel knot. The cells at the periphery of the enamel organ form the outer dental epithelium, and the cells immediately next to the dental papilla form the inner dental epithelium. During the bell stage, special secretory cells start to differentiate. Mesenchymal cells adjacent to the inner dental epithelium and in contact with the basement membrane differentiate into odontoblasts and the cells of the internal dental epithelium differentiate into ameloblasts.

The secretory stage begins when the odontoblasts start to secrete an organic matrix of dentin, mainly collagen. The ameloblasts start to secrete organic matrix of enamel, later to be mineralized, subsequent to dentin formation (Ten Cate, 1985). The odontoblasts move deeper to the centre of dental papilla leaving behind the

growing mineralized dentin layer. Also the ameloblasts move as the enamel thickens, but in the opposite direction than the odontoblasts. Crown morphogenesis is followed by the formation of the roots of the tooth and subsequent crown eruption into the oral cavity (Thesleff, 1997). Cementoblasts originating from dental follicle lay down dental cementum to the outer surfaces of roots. The fibrous periodontal membranes connecting the roots to the alveolar bone originate also from the dental follicle (Thesleff and Sharpe, 1997).

The early stages of tooth development are similar to those occurring in other epithelial organs, such as hair and salivary and mammary glands. In all these, development is regulated by reciprocal and sequential epithelial-mesenchymal interactions that are mediated by signalling networks. More than 300 genes are expressed in the developing tooth (see tooth database http://bite-it.helsinki.fi). The roles of several protein families, such as transforming growth factor β (TGF-β), fibroblast growth factor (FGF), hedgehog (Hh), Wnt, and tumour necrosis factor (TNF) taking part in tooth development, have been intensively studied (Jernvall and Thesleff, 2000; Thesleff and Mikkola, 2002). The earliest interactions regulate tooth initiation, and the last the secretion and mineralization of dentin and enamel as well as root development (Thesleff, 1997). The development of the molars progresses from the first to the third molar (Shellis and Berkovitz, 1981). In each tooth, the development progresses vertically from crown to root (Thesleff, 1997).

Tooth development is under genetic control but can be subjected to environmental disturbances. The consequences of the dysfunctions of mutated genes range from hypomineralization of dentin or enamel to missing of teeth. Several genes are required for normal dental development e.g. *Msx-1*/2, *Dlx-1/2* and *Gli2/3*. Consequently, in knockout mice, lack of these genes results in the arrest of tooth morphogenesis at the initiation or bud stages (Thesleff, 1997; Thomas *et al.*, 1997). As mentioned, dental aberrations can also be due to environmental factors such as exposure to irradiation or chemotherapy or environmental toxicants (Alaluusua *et al.*, 2001).

The development of human and rodent teeth is basically similar (Thesleff and Nieminen, 2001). The main differences are that humans and other primates have two sets of teeth, deciduous and permanent, but rodents have only one set. Additionally, rodents have only incisors and molars whereas humans have incisors, canines, premolars and molars. The molars develop similarly both in humans and rodents species, but rodent incisors grow continuously and in that respect differ from those of humans.

The gestation of rats lasts for about 21 days. The first, second and third lower molars are initiated on gestation day (GD)13, 14-15 and GD20/21, respectively, in rats. Mineralization starts about one week after initiation in the first and second molars,

and two weeks after initiation in the third molars. The molars erupt four weeks after initiation, except for the third molars that erupt five weeks after initiation. The rat first, second and third molars are functionally ready on PND25, 28 and 40, respectively. The lower molars develop approximately one day earlier than their upper counterparts (Shellis and Berkovitz, 1981).

The period of human dental development ranges from early gestation up to late adolescence. The first deciduous tooth is initiated in the fifth, and mineralization starts in the 14th week of gestation (Thesleff, 2003). Initiation of the first permanent teeth occurs at around the 20th week after fertilization (Ten Cate, 1996). The permanent first molars start to mineralize around birth and primary mineralization of the crowns is completed at around the age of three. Further maturation proceeds until the teeth erupt at around the age of six. The last teeth to be formed, the third molars, are initiated after birth and the crown development is completed at the age of 13-14 years (Thesleff, 1997). The development of third molars is ready at around 20 years (Thesleff, 2003). Thus, there are teeth at many developmental stages in young children. The development of deciduous teeth is partly protected against acquired defects as they develop during gestation. However, compounds that cross the placenta can affect the development of deciduous teeth, as can metabolic diseases of the foetus. Permanent teeth are vulnerable to environmental disturbances of systemic or local origin during the entire period of development.

Even though tooth development is initiated before bone formation starts in the jaws, the development of the teeth and the surrounding bones is strictly co-ordinated in its later stages. After birth, the teeth regulate the extent and directions of growth of the jaw bones (Ten Cate, 1985).

2.4 Bones: structure and development

Bone is essentially a mineralized connective tissue found in vertebrates serving structural and metabolic purposes. The skeleton gives form and support to the body allowing movement in conjunction with the action of the muscles. Bones protect the soft tissues of vital organs and also contain the bone marrow. Bones can also be considered as a metabolically active reserve of ions, especially calcium. Cortical and trabecular bone are the two macroscopic bone structures. Cortical bone is a dense solid mass with only small space and channels, and it is located in the shafts (diaphysis) of the long bones. Trabecular bone is structurally sparse and mechanically weaker than cortical bone and is located in the vertebrae, pelvis and ends (epiphyses) of the long bones. Both architectural structures share the same matrix composition (Eriksen *et al.*, 1994). Cortical and trabecular bone differ in their

main purposes: mechanical and protective function (cortical bone) and metabolic function (trabecular bone) (Marks and Odgren, 2002).

As a tissue, bone consists of cells, fibres and extracellular material, or matrix that is hard and calcified. In contrast to another mineralized and acellular tissue, enamel, bone tissue is cellular and active. It possesses a circulation and undergoes constant remodelling, which renews the bone tissue. Remodelling occurs via bone resorption by osteoclasts and by synthesis of new bone matrix by osteoblasts and the subsequent matrix mineralization. Remodelling serves two purposes: maintaining mechanical integrity and releasing or storing calcium according to metabolic needs. Bone modelling, on the other hand, means the change of bone shape to better meet physiological needs or mechanical stress. (Eriksen *et al.*, 1994)

Even though bone is hard and brittle, it does possess some elasticity due to its organic components. Bone tissue consists of 70% inorganic salts, mostly hydroxyapatite and 22% organic matrix and 8% water (Marks and Odgren, 2002). The organic matrix consists of 95% type I collagen with the rest being composed of proteoglycans and numerous noncollagenous proteins (Marks and Odgren, 2002). Bone marrow is a specialized connective tissue closely related to bone. Its main function is to serve as a production site for blood cells and it is found in the medullary cavities of certain long bones and in the spaces of trabecular bone in some areas in adults (Thibodeau and Patton, 1999).

Most bones are derivatives of mesoderm, in addition many skull bones originate from ectomesenchyme (Sadler, 2006). Interactions between epithelium and mesenchyme are required for normal skeletal development in a way similar to that in teeth. These interactions occur before cell migration is completed for most neural crest cells later to form craniofacial skeleton (Hall, 1978; Hall, 1991). The axial skeleton originates from the somites, limb bones from the lateral plate mesoderm and branchial arch and craniofacial bones from the cranial neural crest (Gilbert, 2003). The basic form and development of bones of the skeleton are genetically determined. Bones can be formed *via* either endochondral or intramembranous ossification, but despite these different terms, the resulting bone is basically similar. The terms refer to the sites or environments were the ossification occurs. In endochondral ossification, which occurs in vertebrae, ribs and limbs, minute cartilage models of the bones are formed (Fig 4). The cartilage is not converted to bone, but rather is gradually replaced by bone as the models grow (Olsen, 2003). Mesenchymal cells around the cartilaginous model differentiate into osteoblasts and invade the middle shaft of the model with osteogenic cells and capillaries where they form the centre of ossification. The cartilaginous model increases length by interstitial growth at both sides. The chondrocytes in the middle of the model mature and by virtue of calcification, the intercellular substance become deprived of

nutrients. Thus the chondrocytes die and the central part begins to break up. Ossification starts on the remnants of the cartilaginous model, as osteoblasts, osteogenic cells and capillaries invade the centre of the cartilaginous model and form the ossification centre. Osteoblasts secrete at first intercellular substance and later express membrane-bound alkaline phosphatase, which initiates mineralization. Before calcification the bone consisting of organic matrix is called osteoid. Ossification spreads from the centre and invades the degrading calcified model thus increasing the length of the bony structure forming at the first trabecular bone. The width of the bone grows as new cortical bone is laid along the sides (appositional mechanism). The trabecular bone in the central part is removed and thus the marrow cavity becomes formed in the middle of the bone (Baron, 2003). Interestingly, the original embryonic model of bone retains its shape as it grows bone. This is achieved by well-coordinated cartilage growth, erosion and bone apposition (Alberts *et al.*, 1983).

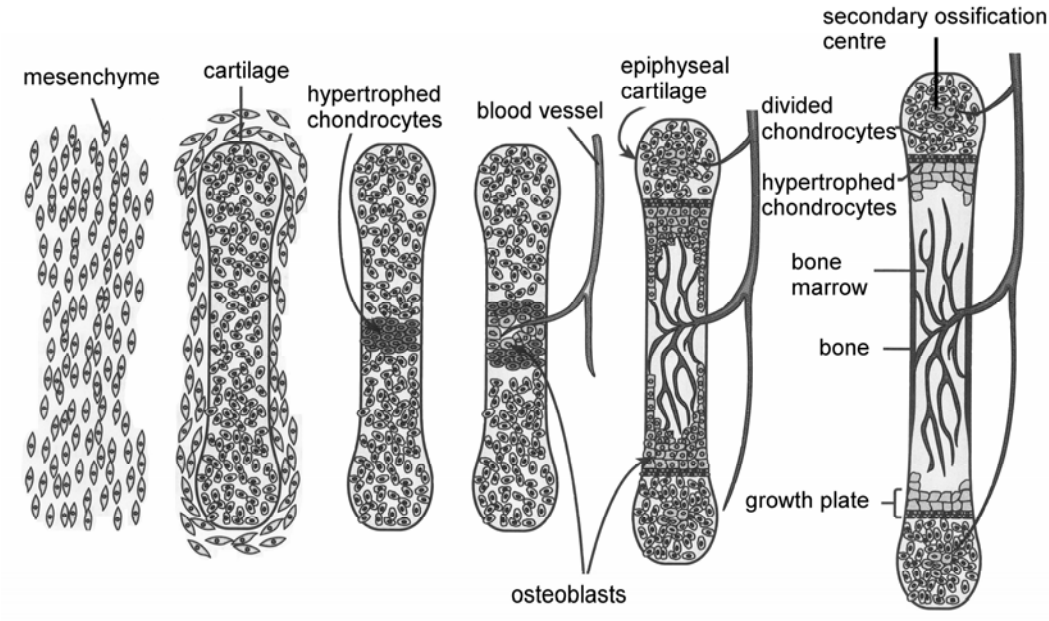

Figure 4. Endochondral ossification (modified from Thesleff and Salminen, 2003 with permission)

Most of the skull bones are formed by intramembranous ossification. These bones normally start developing from two centres of ossification where mesenchymal cells differentiate into osteoblasts. The osteoblasts start to secrete the organic matrix of bone, and those cells embedded in the matrix are transformed into osteocytes (Ham, 1974). The collagen-glycosaminoglycan matrix is capable of binding calcium salts delivered to the region through capillaries (Gilbert, 2003). A population of stem cells resides at the edges of the newly formed bone assuring a steady supply of osteoblasts (Ham, 1974). In flat bones, the original centre of ossification lays down trabecular bone, whereas on the outsides of the bone osteoblasts from the periosteum form virtually avascular bone. The inner trabecular bone is invaded and supplanted by vascular tissue that differentiates into bone marrow (Snell, 1972).

In rats, bone development starts after the midpoint of gestation. The very first sign of bone development is a condensation of mesenchymal tissue on GD13. Cartilaginous structures appear in skeleton on GD15, also known as Meckel's cartilage in the future mandible (Hebel and Stromberg, 1986). Practically all skeletal elements are ossified in rats at term, the last structures to ossify before PND2 are the phalanges and sternebra (Fritz and Hess, 1970) .

In humans, the development of bones starts during the third month of gestation and lasts up until 25 years. Ossification of the auditory ossicles begins in the fourth month of gestation and these are the first bones to become fully ossified (Sadler, 2006). Incomplete ossification prevents damage to children during birth and fractures in toddlers with lots of energy and lack of skills. In adults, density of skeletal structures permits mechanical stress (Thibodeau and Patton, 1999).

2.5 Regulation of bone development

Bone development and later remodelling are regulated by complex control systems. The signal mediators affecting osteoblast and osteoclast differentiation include transcription factors, growth factors and hormones. Transcription factors regulate the expression of specific genes and thus the expression of cell specific proteins, but only a few which are important for osteogenesis will be described here. Cbfa1 expression in embryos is limited to prospective chondrocytes or osteoblasts and it is the earliest and the most specific marker of osteogenesis. Mice deficient for *Cbfa1* are born with normally patterned skeleton that consists exclusively of cartilage (Ducy *et al.*, 2000). Transcription factors Runx2 and Osterix are involved in the initial condensation of mesenchymal cells in intramembranous ossification (Baron, 2003). Vitamin D ($1,25(OH)_2D_3$) regulates gene transcription at a later stage of osteoblast differentiation (Lian *et al.*, 2003), but also retinoic acid is required for normal skeletal development. Osteoclast differentiation requires at least two

transcription factors, PU-1 and MiTf, during its early stages (Baron, 2003). Subsequently other transcription factors such as c-Fos, NF- κB and M-CSF, to name a few, are required for osteoclastogenesis (Teitelbaum, 2000). An essential protein for osteoclastogenesis and for regulation of mature osteoclasts activity is RANK ligand (RANKL) that is secreted by osteoblasts. As RANKL interacts with RANK in preosteoclasts, the precursor cells differentiate into osteoclasts with the condition that also macrophage colony-stimulating factor (M-CSF) is present (Jüppner and Kronenberg, 2003).

Growth factors affect the replication and differentiation of undifferentiated cells. They can also change the differentiated function of mature cells and thus regulate bone remodelling (Canalis, 2003). Examples of the growth factors regulating bone development are fibroblast growth factors (FGFs), bone morphogenetic proteins and insulin-like growth factors (Canalis, 2003; Lian *et al.*, 2003). Hormonal regulation of skeletogenesis involves parathyroid hormone related protein (Lian *et al.*, 2003). Bone growth and remodelling are influenced by oestrogens (possibly inhibit osteoclastic bone resorption), leptin (inhibits bone formation by affecting differentiated osteoblasts), PTH (stimulates bone formation and osteoclastic bone resorption) and calcitonin (inhibits osteoclastic bone resorption and osteoclasts formation) (Ducy *et al.*, 2000; Mundy *et al.*, 2003). Additionally, the glycoproteins and proteoglycans in the ground substance are believed to play an important role in the calcification process (Baron, 2003).

It has to be noted that osteoblasts influence the differentiation of osteoclasts and furthermore, the osteoclasts affect the osteoblasts during bone remodelling (Marks and Odgren, 2002). The importance of the interactions is illustrated by the fact that receptors for some osteoclastic factors are expressed in the osteoblasts, not in the osteoclasts (Lian *et al.*, 2003). Osteoclast differentiation requires the presence of marrow stromal cells or their osteoblast progeny (Teitelbaum, 2000).

Local factors as well as systemic factors regulate bone remodelling (Mundy *et al.*, 2003). Subsets of osteoblast lineage cells respond differentially to signals (Lian *et al.*, 2003) and bone remodelling occurs in distinct areas throughout the skeleton (Mundy *et al.*, 2003).

2.6 Effects of food deprivation on bones and teeth

Starvation has adverse effects on bones in humans and in rats. Females with self induced starvation (anorexia nervosa) exhibited significantly reduced bone mineral densities compared to that of healthy controls (Jacoangeli *et al.*, 2002). It is possible that restricted feed intake results in delayed bone maturation. Rats deprived from

nutrients prenatally between GD17-birth or with restricted postnatal food supply after birth had a smaller total body bone mineral content (TBBMC) than controls at puberty and at the age of six months. However, when TBBMC was adjusted for the body weight, the difference disappeared, indicating that TBBMC was clearly related to body weight (Engelbregt *et al.*, 2004). Feed restriction to 60% of *ad libitum* level beginning at the age of 6 weeks reduced bone strength, weight and ash weight in rats studied at the age of 4 and 13 months. Once again, the ratio between bone weight and body weight ratio was similar between the groups (Nnakwe, 1998). In adult rats, food deprivation altered serum levels of calcium and potassium ions and upregulated the expression of the mRNA levels of PTH and PTH/PTHrP receptors in bones (Kawane *et al.*, 1997).

The effects of malnutrition on dental development have not been studied exhaustively. Early malnutrition affects tooth formation and increases the incidence of dental caries in later years (c.f. Alvarez *et al.* 1988). In experimental rats, a limited supply of protein during suckling period has decreased tooth and bone weight in the offspring (DiOrio *et al.*, 1973). Chronic malnutrition delayed primary tooth exfoliation in Peruvian children and increased the incidence of caries in children older than 6 years (Alvarez *et al.*, 1988). Malnutrition may result in increased caries susceptibility attributable to the appearance of enamel defects, such as linear enamel hypoplasia (Seow, 1998). Theoretically, food deprivation in adulthood could only affect dental pulp tissue and the odontoblasts lining the dental pulp, since other elements of tooth are not viable. However, malnutrition may affect the mineral composition of saliva and thus interfere with mineral exchange between enamel and saliva. This could affect the enamel mineral composition and its resistance to caries.

2.7 Comparison of tooth and bone

Bones and teeth develop from mesodermal cells, with the exception of enamel which is formed of ectodermal cells. The composition of dentin is similar to that of bones (Thesleff, 1997), and odontoblasts and osteoblasts resemble each other, but in the dentin there are only cytoplasmic processes of odontoblasts whereas bones are sparsely populated with osteocytes. Enamel is acellular and inactive tissue; the ameloblasts residing on the enamel surface become destroyed as the tooth erupts. The bones are constantly renewed by the action of osteoclasts and osteoblasts (remodelling), whereas teeth are not remodelled once they have formed. However, secondary dentin can be laid onto the inner surface of the dentin pulp thus decreasing the size of the pulp. Rodent incisors grow constantly as opposed to human and rodent molars that do not grow once they have achieved their mature

size. Bones have veins nourishing the osteocytes, whereas in teeth circulation and nerves are found only in dental pulp.

Initiation and early morphogenesis of teeth occur before bone formation starts in the jaws. However, the development of the teeth and the surrounding bone is strictly co-ordinated. The development of these mineralized tissues depends on the epithelial-mesenchymal interactions. After birth, the teeth regulate the extent and direction of the growth of the alveolar processes of the jaw bones (Ten Cate, 1985). In endochondral ossification, a cartilaginous template is initially formed by chondroblasts whereas no cartilage is involved in the development of teeth. One special distinction between rat and human bone development is that as a human being reaches skeletal maturity, the growing zone of cartilage (metaphysic or growth plate) ossifies and the growth in length-wise terminates (Thibodeau and Patton, 1999), but an inactive growth plate remains present in aged rats (Roach *et al.*, 2003).

2.8 Effects of PCDD/Fs and PCBs on tooth and bone in animals

2.8.1 Studies in adult animals

The literature dealing with the effects of PCDD/Fs or PCBs on bone and teeth is relatively scarce. It is known that exposure to high doses of TCDD or PCBs evokes bone alterations in laboratory animals. Near lethal dioxin-like PCB exposure administered *via* the diet induced osteolysis in maxilla and mandible of juvenile minks (Render *et al.*, 2000). A single TCDD dose of 1000 µg/kg administered to young adult males of the resistant rat strain, H/W (*Kuopio)* with both dioxin resistance alleles, impaired skull growth, resulting in smaller skull size 16 weeks after dosing. In the same study, TCDD caused defective dentin formation and pulpal perforation of continuously erupting incisor teeth (Alaluusua *et al.*, 1993). Hexachlorobenzene (HCB, a non-dioxin like organochlorine pesticide) induced maxillary incisor degeneration in rats after prolonged exposure (Long *et al.*, 2004). Cystic periodontal lesions around the unerupted tooth were found in rhesus macaques after a high dietary exposure to Aroclor 1248, a PCB mixture containing mainly tetra-, penta- and tri-PCBs (McNulty, 1985).

Chronic exposure to TCDD can cause alterations in bone geometry, i.e. a decrease in tibial length and cross-sectional size and reduced breaking force and stiffness of tibia in the two rat strains with different TCDD sensitivity; the resistant H/W (*Kuopio*) and the sensitive L-E (*Turku/AB*). Even though H/W rats with the mutated AHR and the other resistance allele *B* were more resistant than L-E rats, the sensitivity difference was only about 10-fold (Jämsä *et al.*, 2001), whereas the

difference is about 1000 fold with respect to acute lethality (Pohjanvirta *et al.*, 1987; Tuomisto *et al.*, 1999). In the same study, the lower incisors were affected in both strains without there being any clear sensitivity difference. The aberrations involved odontoblastic and pulpal cell death accompanied by arrested dentin formation. Furthermore, precocious squamous metaplasia was found in the postsecretory enamel organ (Kiukkonen *et al.*, 2002). These results indicate that the incisor defects could be classified in the type I category whereas bone defects might belong to type II endpoints.

In adult female rats, coplanar PCB126 reduced bone length, water content and torsional stiffness (Lind *et al.*, 2000). In ovariectomized rats the exposure decreased tibial length and increased bone mineral density. Sham-operated animals responded to exposure in a manner different from intact animals: PCB exposure increased osteoid surface, cortical thickness and organic content. (Lind *et al.*, 1999).

2.8.2 Studies in developing animals

PCB exposure has been shown to arrest bone growth in birds. American kestrels, exposed after hatching to doses of PCB126 that did not significantly decrease body weight, exhibited significantly shorter crown-rump length and long bone length in the appendages (Hoffman *et al.*, 1996). Eggs laid by hens that had consumed a diet containing PCB contaminated carp were artificially hatched and studied. The higher exposure level (59 ng TEQ/kg feed) resulted in decreased hatchability in association with feet and leg deformities as well as in malformed brain cases and poorly ossified skull bones in the embryos and chicks (Summer *et al.*, 1996).

Developmental studies have revealed that TCDD affects cartilage, bone and tooth development in fish. TCDD reduced the length of craniofacial structures as well as total length of developing rainbow trout (Hornung *et al.*, 1999; Carvalho *et al.*, 2004), and caused severe dysmorphogenesis in craniofacial structures of zebrafish (Mattingly *et al.*, 2001). TCDD also disrupted calcification in spinal cord and spines in medaka fish (Kawamura and Yamashita, 2002), retarded rib development in rainbow trout (Hornung *et al.*, 1999) and altered vertebral development in zebrafish (Mattingly *et al.*, 2001). An absence of teeth was reported in exposed sac fry of rainbow trout 47 days after fertilization (Hornung *et al.*, 1999).

Very few developmental studies have reported the effect of TCDD on bones or teeth in mammals. A single maternal dose of 0.5 µg/kg or 1.0 µg/kg on GD9 affected the shape and reduced the size of the mandibles in mouse offspring (Allen and Leamy, 2001). In mice, lactational exposure hastened incisor eruption (Madhukar *et al.*, 1984). The development of interfrontal bone was retarded in embryonic mice after a

maternal dose of 25 µg/kg TCDD on GD10 (Peters *et al.*, 1999). High level maternal exposure to a mixture of dioxin-like and non-dioxin-like PCBs and organochlorines, excluding PCDD/Fs, mimicking the mixture found in the blood of Canadian Great Lake residents, resulted in facial malformations, especially a rounded skull and underdeveloped snout and lower jaw. Another studied PCB mixture that contained only non-dioxin like PCBs (Aroclor 1254) did not induce these defects (Bowers *et al.*, 2004). No effect of TCDD was seen in the first and second molars of embryonic mice on GD18, the third ones are not observable at that time point (Miettinen *et al.*, 2004). TCDD exposure during gestation and lactation has interfered with the development of dentition in rhesus monkeys. A maternal dose of 0.3 µg/kg on GD20 with maintenance doses of 15 ng/kg every 30 days during pregnancy and lactation resulted in incomplete calcification, accelerated eruption of teeth as well as malshaped and missing teeth in the offspring. *In utero* exposure without lactational exposure was sufficient to induce these effects, as seen in the stillborn offspring (Yasuda et al., 2005a). Preliminary results indicate that the same dose can prevent the development of the third molars (Yasuda et al., 2005b). Yasuda *et al.* have not yet reported the possible effects of TCDD on bone development in their study. On the other hand, rhesus and cynomolgus monkey neonates, exposed after birth to a similar PCB mixture via bottle feeding as Canadian breast-fed infants receive *via* breast milk, exhibited no anomalies in their skeletal development or tooth eruption. The mixture contained 15 PCB congeners, of which 10 were non-dioxin like with a WHO TEF 0, and five were dioxin-like congeners (2 with TEF 0.0005 and 3 with TEF 0.0001) (Arnold *et al.*, 1999). Administration of a high dose of TCDD (1000 µg/kg) to lactating H/W rat dams on PND1 blocked the development of offspring third molars, halted molar root formation and arrested dentinogenesis of the incisors (Lukinmaa *et al.*, 2001). In addition, enamel maturation in the molars was impaired and dentin mineralization retarded. TCDD caused a dose-dependent decrease in organic matrix degradation in enamel, this being a possibly permanent defect (Gao *et al.*, 2004). Thus, at least in rats, it seems that TCDD can completely block the development of the third molars, whereas the incisors respond with deviated dentinogenesis and pulpal perforations (Alaluusua *et al.*, 1993; Lukinmaa *et al.*, 2001; Kiukkonen *et al.*, 2002).

2.8.3 Wild-life studies

Some reports have linked the lesions observed in top-predator animals with environmental exposure to organochlorines. Very high concentrations of dioxins and related compounds, up to 40 000 TEQ pg/g fat wt, have been detected in birds and marine mammals living in polluted areas (Tanabe, 2002). Baltic seals sampled during 1960-1985 - a period of heavy organochlorine contamination of the Baltic

Sea - suffered from severe bone loss in skull (Bergman *et al.*, 1992). Another study linked periodontitis and tooth loss, as well as other lesions, in beluga whales from the St Lawrence Estuary with organochlorine exposure (De Guise *et al.*, 1995). The bone mineral density in East Greenland polar bears correlated negatively with the subcutaneous adipose tissue concentration of organochlorines, for example ΣPCB (Sonne *et al.*, 2004). Environmental exposure to organochlorines, such as DDE, a metabolite of the pesticide DDT, can reduce reproductive success in wild birds via eggshell thinning. The levels of PCBs continued to correlate with poor productivity even when the DDE levels decreased below the critical level to cause eggshell thinning (Bowerman *et al.*, 1995). Artificially hatched eggs of terns collected from the heavily organochlorine contaminated Great Lakes suffered from low hatchability, growth-retardation, and at autopsy, isolated cases of weak ossification and shortened mandibles, even lack of jaws or skull bones were found in the chicks (Gilbertson *et al.*, 1991).

2.8.4 *In vitro* studies

TCDD hampers osteogenesis also *in vitro*. Alkaline phosphatase activity, a marker of the osteoblastic phenotype and bone formation, has been reduced by TCDD in three cell models (Gierthy *et al.*, 1994; Singh *et al.*, 2000) followed by dramatically impaired mineralization (Singh *et al.*, 2000). TCDD impaired postproliferative osteocalcin expression and reduced multicellular nodule formation, important in bone tissue-like organization, by normal rat diploid osteoblasts (Gierthy *et al.*, 1994). In line with these studies, preliminary results from our laboratory suggest that TCDD exposure hampers matrix maturation and its mineralization (Natunen *et al.*, 2005). Even though AHR was expressed more in osteoclasts than in other cells derived from bone marrow, TCDD did not increase bone resorption since neither the number nor the activity of mature osteoclasts was changed after TCDD exposure (Ilvesaro *et al.*, 2005a). However, osteoclastogenesis was severely inhibited by TCDD (Ilvesaro *et al.*, 2005b).

Treatment with TCDD disrupts also dental development *in vitro*. The defects include depolarization of secretory odontoblasts and ameloblasts with consequent failure of dentin matrix mineralization, lack of enamel matrix deposition and concomitant alteration of cuspal morphology in murine embryonic teeth (Partanen *et al.*, 1998). TCDD can also arrest tooth development *in vitro* given that the exposure starts at the initiation stage. Later stage exposure results in smaller tooth size and disrupted cuspal morphology (Partanen *et al.*, 2004b).

2.9 Effects of dioxins and dioxin-like chemicals on human teeth and bone

2.9.1 Cases of accidental exposure: Yusho, Yucheng, Seveso

Exposure to high dose of PCBs and dioxin-like chemicals has evoked oral lesions in humans. Two similar mass PCB poisonings of humans occurred in Japan and Taiwan in 1968 and 1979, the Yusho and Yucheng incidents, respectively. In both cases, the exposures were attributable to contaminated cooking oil. In Japan, the oil contained PCBs, PCDD/Fs, polychlorinated terphenyls and polychlorinated quarterphenyls; in Taiwan the oil was mainly contaminated with PCBs and PCDFs. Japanese adults suffered from various symptoms, such as acneiform skin eruptions, hyperpigmentation of skin and membranes, increased discharge of the eyes. The disease was named Yusho (oil) disease. Affected mothers gave birth to dark coloured babies that had natal teeth. Some of these babies had large and wide openings in frontal and occipital fontanels and the sagittal suture was wider than normal. Additionally, the calcification of the parieto-occipital area of the skull was disturbed (Hamada, 1996). Broken teeth were found more often in patients exposed at age over 16 than in nonexposed subjects (Guo *et al.*, 1999). In Taiwan, the syndrome was called Yucheng disease, and the symptoms involved, chloracne and hyperpigmentation in adults. Nine percent of transplacentally exposed children had natal teeth, whereas none of the control subjects exhibited this trait. At physical examination, tooth chipping was found in 11% of the patients as opposed to control value 0% and caries in 68% vs 54% in exposed and control children, respectively (Rogan *et al.*, 1988). Other studies revealed that Yucheng children had abnormally shaped tooth roots, the eruption of permanent teeth was retarded and there was a high frequency of missing permanent teeth germ, this occurring more often in girls than in boys (Lan *et al.*, 1989).

Exposure to pure TCDD occurred in Seveso, Italy, in 1976 after a chemical factory explosion. A substantial amount of TCDD – estimates range from hundreds of grams up to 34 kilograms - was released and several thousand people were exposed (Bertazzi *et al.*, 1998). Missing permanent teeth (lateral incisors and second premolars) have been recorded in Seveso subjects that were less than 9.5 years old at the time of the incidence. In addition, occurrence of missing permanent teeth and developmental defects of enamel correlated with the extent of TCDD exposure. Gingival pigmentation, periodontal disease and caries were not associated with TCDD exposure (Alaluusua *et al.*, 2004).

2.9.2 Occupational, environmental and dietary exposure

Mothers working in a capacitor factory and who were exposed to PCBs reported carious teeth more often in their children who had been breast-fed for more than 5 months. Some children exhibited gingival pigmentation and mottled enamel (Hara, 1985). It is noteworthy, that the results of human caries susceptibility after accidental or occupational exposure are inconsistent (Hara, 1985; Rogan *et al.*, 1988; Alaluusua *et al.*, 2004). Children living in a PCB contaminated area in Slovenia had more developmental defects of enamel (mainly demarcated opacities and hypoplasia) than controls. The teeth that were most frequently affected were the incisors and premolars (Jan and Vrbic, 2000).

Background exposure to PCDD/Fs via mothers' milk in 1987 correlated with enamel hypomineralization of the first permanent molars that mineralize for the most part during the first 2 years of life. An important factor was the total exposure, this being dependent on the concentration of the milk and the duration of lactation, which correlated with both the frequency and severity of the lesions (Alaluusua *et al.*, 1996b). The defects correlated clearly with the total exposure to PCDD/Fs, but only weakly with PCB exposure (Alaluusua *et al.*, 1999). Another Finnish study linked the duration of lactation with mineralization defects in children born in 1981-1984 (Alaluusua *et al.*, 1996a). At the prevailing background exposure levels, PCDD/Fs and PCBs did not correlate with the occurrence of perinatally erupted teeth in Finland (Alaluusua *et al.*, 2002).

No effects of high exposure to PCBs and dioxin-like chemicals on bones of adults have been reported after Yusho, Yucheng or Seveso accidents. Exposure to the organochlorines with hormone-like properties could be predicted to alter bone composition. However, the results on the effect of DDE on bone mineral density have been very inconsistent (Beard *et al.*, 2000; Bohannon *et al.*, 2000; Glynn *et al.*, 2000). An epidemiological study of Swedish fishermen and their wives suggested that those living next to the organochlorine contaminated Baltic Sea would have an increased risk for vertebral fractures as compared to those from the less contaminated Swedish west coast. However, the smoking incidence, a risk factor for osteoporosis, differed between exposed and controls, and individual exposures were not determined (Alveblom *et al.*, 2003). A more detailed study provided limited support to the earlier result proposing that females from the coastal regions of the Baltic Sea eating more than one fatty fishmeal each month had greater risk for osteoporotic fractures than those living on the west coast (Wallin *et al.*, 2004).

2.10 The role of AHR in development of hard tissues

AHR and ARNT are expressed in several embryonic tissues including bone and teeth (Abbott *et al.*, 1995; Mattingly *et al.*, 2001; Sahlberg *et al.*, 2002). AHR and ARNT are usually expressed simultaneously in the same or adjacent tissues; therefore it is plausible that the expression is regulated in a co-ordinated manner (Abbott *et al.*, 1995; Abbott and Probst, 1995). AHR is not essential for embryonic development, since viable $AHR^{-/-}$ mice are born at the expected frequency from heterozygous matings (Fernandez-Salguero *et al.*, 1995). However, only about every second $AHR^{-/-}$ mouse survives to adulthood and those who do grow slower, have reduced retinoic acid metabolism, smaller livers, cardiomyopathy, epidermal hyperplasia, vascular hypertrophy, as well as other lesions (Fernandez-Salguero *et al.*, 1995; Andreola *et al.*, 1997; Fernandez-Salguero *et al.*, 1997). An embryonic structure in liver, the ductus venosus, fails to close, which results in the formation of a portocaval vascular shunt in $AHR^{-/-}$ mice (Lahvis *et al.*, 2000)

AHR is also not required for skeletal development. The bones of AHR-knock out mice developed rather similarly to those of their wild type littermates with the exception that $AHR^{-/-}$ mice a had smaller frequency of large interfrontal bones (Peters *et al.*, 1999). Mis-timed expression of AHR caused by dioxins has been linked with developmental difficulties. TCDD-induced expression of AHR in nose and vertebrae preceded morphologic abnormalities in these structures in developing zebrafish (Mattingly *et al.*, 2001).

Bone cell cultures from the bone marrow showed that both osteoblasts and osteoclasts express AHR (Ilvesaro *et al.*, 2005a). It is interesting that an AHR ligand, 3-methylcholanthrene, can retard proliferation and differentiation of osteoblasts *in vitro* and ossification of mouse embryos *in vivo* (Naruse *et al.*, 2002). Furthermore, resveratrol (3,5,4'-trihydroxystilbene), a pure AHR antagonist, has reversed TCDD-induced inhibition of osteodifferentiation (Singh *et al.*, 2000). Therefore it is apparent that the effects of TCDD on bone are mediated, at least in part, *via* AHR.

Both ameloblasts and odontoblasts express AHR in embryonic mice and juvenile rats (Sahlberg *et al.*, 2002; Gao *et al.*, 2004). The expression depends on the developmental stage (Sahlberg *et al.*, 2002), e.g. TCDD has depressed the expression of AHR in rat molar ameloblasts and odontoblasts when given on PND22, whereas it had no effect on the expression on PND9 (Gao *et al.*, 2004). Reports on AHR knock out mice have not described any disturbances in dental development (Fernandez-Salguero *et al.*, 1995; Andreola *et al.*, 1997; Fernandez-Salguero *et al.*, 1997).

3 AIMS OF THE STUDY

PCDD/Fs cause a wide spectrum of harmful effects in both adult and developing animals. We used TCDD as a model compound to study whether PCDD/Fs could affect hard tissue development. TCDD is a teratogenic agent inducing structural malformations such as cleft palate (Pratt *et al.*, 1984) as well as disturbances in sexual organ development and sexual behaviour at very low doses (Mably *et al.*, 1992a; Mably *et al.*, 1992b; Mably *et al.*, 1992c; Gray *et al.*, 1997a; Gray *et al.*, 1997b; Hamm *et al.*, 2000). However, there was only meagre knowledge of TCDD-induced tooth and bone defects before initiation of the present studies. It had been shown in mice and rats that TCDD disturbed normal tooth development (Alaluusua et al., 1993; Partanen et al., 1996; Lukinmaa et al., 2001), but no experimental dose-response studies were available. These were considered important since clinical observations suggested that PCDD/F exposure via mothers' milk was associated with mineralization defects in the child's molars (Alaluusua *et al.*, 1999). Furthermore, a high dose of TCDD has impaired skull growth and mineralization (Alaluusua *et al.*, 1993) and long-term treatment at low doses impaired the geometry, mineral density and mechanical strength of long bones in young adult rats (Jämsä *et al.*, 2001). Therefore it was of interest to clarify whether low prenatal doses could affect bone development in rats. Structural strength tests and geometry studies were performed on cortical bones due to methodological reasons.

Specific aims were as follows:

I To study if TCDD at low doses can affect tooth development in rats and to test whether the resistance alleles Ahr^{hw} and B^{hw} can alter the sensitivity to developmental tooth defects

II To determine the critical time of sensitivity for developmental tooth defects

III To investigate whether TCDD can disturb bone development in rats, to study if the resistance alleles Ahr^{hw} and B^{hw} are associated with the sensitivity to developmental bone defects, to study if the bone defects are reversible in rats and to measure TCDD levels in dams and offspring

IV To elucidate whether prenatal TCDD exposure can enhance the susceptibility to caries in rats

4 MATERIALS AND METHODS

4.1 Animal care

Line A, B and C rats were obtained from the SPF barrier unit of the National Public Health Institute (Kuopio, Finland). Regular health surveillances consisting of bacteriological and serological screening as proposed by FELASA (FELASA, 1996; FELASA, 2002) indicated that the animals were free of typical rodent pathogens. The rats were kept under a photoperiodic cycle of 12 h light / 12 h dark (lights on 07:00-17:00 h) in an air-conditioned room. The mean temperature and the relative humidity were 21 ± 1°C and 50 ± 10%, respectively. The temperature and relative humidity were monitored continuously by sensors in each animal room connected to a computer based Honeywell XBSI system (Honeywell, Morristown, USA). Pelleted rat feed (R36, Lactamin, Stockholm, Sweden) and tap water were available *ad libitum*, except for the caries study **(IV)** where the weaned offspring had free access to powdered rat feed (R36, Lactamin, Stockholm, Sweden) containing 15 % sugar and to tap water containing 7 % sugar. Before mating, females were kept in stainless steel cages (bottom 42.0 cm * 24.5 cm, height 15.0 cm) containing aspen-chip bedding (Tapvei Co, Kaavi, Finland), 2-5 rats per cage and covered with wire-mesh lids. After mating overnight (i.e. one male with 1-2 females), the copulated dams were housed individually in Macrolon® polycarbonate cages (bottom 36.0 cm * 19.5 cm, height 15 cm) with aspen-chip bedding. After the midpoint of gestation, the pregnant dams were given paper handtowels for nest material. After weaning on PND21 or 28 offspring were kept in Macrolon® cages similarly to their dams, except for the caries study rats **(IV)**, which were kept singly in wire-mesh cages (bottom 38 cm *20.5 cm, height 19.5 cm). Any material possibly contaminated with TCDD (e.g. bedding, animal excreta and carcases) was collected and sent to Ekokem Oy Ab hazardous waste treatment plant (Riihimäki, Finland) for proper disposal by incineration.

All animal study protocols were reviewed and approved by the Animal Experiment Committee of the University of Kuopio and the Kuopio Provincial Government.

4.2 Chemicals

2,3,7,8-Tetrachloridibenzo-p-dioxin (TCDD) was purchased from the UFA-oil institute, UFA, Russia and it was over 99% pure as confirmed by gas chromatography-mass spectrometry. After weighing, TCDD was dissolved in diethyl ether (BDH Laboratory Supplies, Poole, UK) for storage. To prepare a

dosing solution, the appropriate volume of diethyl ether was mixed with corn oil (Sigma Chemicals, St. Louis, MO) and the ether was allowed to evaporate. Dosing solutions were mixed in a magnetic stirrer and sonicated for 20 minutes before dosing.

4.3 Experimental designs

Female rats were given a single dose of TCDD p.o. (0.03 – 1 µg/kg, 4 ml/kg) either during pregnancy or after parturition. In studies **I** and **IV**, the pregnant rats were exposed to TCDD on GD15. In study **II**, the dosing day was GD11, GD13, GD15, GD19, PND0, PND2 or PND4. Controls received corn oil at volume of 4 ml/kg on GD15. In the cross-fostering study **(II)**, offspring from dams exposed to TCDD on GD15 were transferred to control dams and vice versa on PND0 before the start of lactation. In that way, the offspring were exposed to TCDD only during gestation, via lactation or both. Study **III** was accomplished with samples from studies **I** and **II**. In the caries study **(IV)**, the weaned offspring were given 0.5 ml *Streptococcus mutans* –containing broth placed in their mouth on PND21, 22 and 40, and the controls were given the same volume of broth without the bacteria. A subset of rats were monitored twice a week for feed and water consumption in the caries study. The offspring were killed by CO_2 asphyxiation and cervical dislocation. The days of killing were PND35 (females in dose-response study; **I** and **III**), PND40 (both sexes in time-course study; **II** and **III**), PND70 (males in dose-response study; **I** and **III**), PND77 (both sexes in caries study; **IV**), PND182 (females in the long-time follow-up study; **III**) or PND365 (females in the long-time follow-up study; **III**). Appropriate samples were collected in each study. The skulls were preserved in formalin in the dose-response study and time-course study (**I, II** and **III**) or 100% ethanol in the caries study **(IV)**. The hind limbs were dissected and frozen at –20 °C.

4.4 Radiographs and measuring the molar size

The jaws were radiographed to study the presence of mineralization of dentin and enamel of the lower and upper third molars and to measure molar size. Radiography was carried out using two experimental X-ray units (Phoenix, Radiante Oy, Finland and Iontomat Tridoros, Siemens, Germany). The lower jaws were split into halves and radiographed with their buccal sides facing the film cassette. The upper jaws were radiographed in an axial projection, teeth facing the surface of the film cassette. The radiograph protocols are described in depth in the original publications **(I, II)**.

To measure the mesio-distal length (MDL) at the dentin-enamel borderline the radiographs were digitized in the study **I** and MDL was analyzed with MCID M5+

image analysis system (Imaging Research Inc., Brock University, St. Catharines, Canada). In study **II**, MDL was measured from the radiographs using a stereomicroscope equipped with a scale. It is worth noting that the second and third molars project at an angle, but the angle is similar between the same molars and results between each molar are comparable.

4.5 Bone densitometry

The bones were scanned with a Stratec XCT 960 A pQCT system using software version 5.21 (Norland Stratec Medizintechnik GmbH, Pforzheim, Germany). The bones were inserted into a plastic tube filled with 0.9% NaCl to position the samples for the measurements. A voxel size of $0.148 \times 0.148 \times 1.25$ mm^3 was used for the measurement. Attenuation threshold value of 0.7 cm^{-1} specified the cortical bone. One cross-sectional slice from each bone was scanned at midshaft, which was determined from the scout view of the pQCT system. Parameters measured at the midshaft of the bone were cross-sectional area of cortex (CSA), cortical bone mineral density (BMD), polar cross-sectional moment of inertia (PMI) and periosteal and endosteal circumferences (PERI and ENDO, respectively).

4.6 Mechanical testing

The bones were subjected to mechanical testing after the pQCT measurements. A material testing machine with amplifier and force sensor (Gefran TU K5D, 0-50 kg, Gefran Sensori, Provaglio D'Iseo, Italy) was used to measure the failure load of the three-point bending strength of tibia, femur and the femoral neck. In the three-point bending test, the bone was placed on a support structure with two loading points, 13 mm apart from each other. The pressing force was directed vertically to the midshaft of the bone. To measure the failure load of the femoral neck, the proximal half of the femur was placed axially into a suitable hole on the support structure and pressed in a direction parallel to the femoral shaft. A constant compression speed of 0.155 mm/sec was used in both configurations. A laboratory plotter (Yokogawa LR 102, Yokogawa Europe) recorded the compression load at different time points. The load-deformation relationship was obtained by conversion of the load-time curve. The maximal load (N) was used for evaluation of bone strength and the stiffness (N/mm) was calculated according to the slope of the linear part of the curve.

4.7 Analysis of TCDD tissue concentrations

In study **III**, pregnant rats were exposed to 0.5 µg/kg TCDD, administered in a volume of 4 ml/kg on GD8, GD11 or GD15, 3-5 rats per group. On GD22 females were monitored every half an hour and offspring born were moved from the cage to prevent suckling. Dams and offspring were killed and sampled after parturition was completed, with the exception of one group exposed on GD15 that was allowed to rear offspring until PND5. The offspring were frozen whole at -20°C until homogenization with Bamix M133 mixer (ESGE AG, Mettlen, Switzerland). Each litter was homogenized to gain a litter sample. An identical amount of homogenized offspring tissue from each litter was then pooled to gain a group sample. A similar amount of perirenal adipose tissue from each dam was pooled to obtain a group fat sample. Tissue samples were freeze-dried and extracted in a Soxhlet apparatus with toluene for 18 hours. The solvent was evaporated and replaced with hexane and the fat percentage was determined gravimetrically.

The extract or an aliquot was spiked with an internal standard solution containing ^{13}C-labeled TCDD and was purified using silica gel, carbon and aluminium oxide columns (Vartiainen *et al.*, 1995). Prior to analyses of TCDD by gas chromatography/mass spectrometry (GC/MS), the purified extract was spiked with a recovery standard solution containing ^{13}C-1,2,3,4-TCDD and was concentrated in a nitrogen flow to a final volume of 30 µl nonane. GC/MS analyses were carried out with a VG 70-250SE high resolution mass spectrometer (VG Analytical, Manchester, UK) interfaced to a HP 6890 high resolution gas chromatograph (Hewlett-Packard, Palo Alto, California, USA). The mass spectrometer was operating in the selected ion monitoring (SIM) mode at a resolution of 10.000 in electron impact ionization (EI) mode (35 eV). Two ions of the molecular ion cluster (M^+ and $(M+2)^+$) were recorded for each compound to be studied. Identification of 2,3,7,8-TCDD was verified by a comparison of the GC retention time and ion ratios with those of the reference compound. Detection limits were: liver 0.1 pg/g fresh weight, lipid tissue 0.5 – 5 pg/g lipid weight.

4.8 Caries detection

Dental caries was scored on the mandibular molar hemisections of all animals in the caries study **(IV)**. After the jaws were removed, they were cleaned free of soft tissues and preserved in absolute ethanol. The jaws were hemisectioned sagittally using a diamond blade (Oriola, Espoo, Finland) attached to a dental drill (W&H Dentalwerk Bürmoos GmbH, Austria) and stained with Schiff's reagent for caries scoring according to König (König, 1966). One observer (HMM) scored the fissure

lesions on one side of the mandible without knowledge of the animal treatment. In order to check the reliability of the procedure, another observer did the same for the other side of the mandible on one subset of samples. The repeatability of the observations proved to be good. Two grades of severity were used to classify the lesions of fissure caries; in T-lesions, caries had reached the dentinoenamel junction, and in B-lesions, the dentin was already involved.

4.9 Enamel mineral composition

Relative enamel mineral composition of the lower third molars was studied in the rats in the cariogenic study **(IV)** and from line C rats in the dose-response study **(I)** using either electron probe micro-analyzer (EPMA) or scanning electron microscopy (SEM) and energy dispersive spectrometry (EDS), respectively. The organic material was removed, and the jaws were fixed in 10 % neutral formaldehyde **(I)** or in 99% ethanol **(IV)** at room temperature. For SEM the third molars were dissected and dehydrated through a graded ethanol series. The third molars from both studies were embedded in plastic (EpoFix Kit, Struers, Denmark) according to the manufacturers instructions using brass ring as a mould. The molars were ground transversally through the crown in order to obtain both enamel and dentin in the ground section. The surface was polished with sanding papers (Exakt, Oy Algol Ab, Espoo Finland) up till grit 2000 **(IV)** or 4200 **(I)** and the surface was coated with carbon.

Electron probe micro-analyzer (EPMA)

Jeol JXA-8200 superprobe WDS/EDS electron probe micro-analyzer (EPMA, Jeol, Japan) with five spectrometers and 10 crystals was used to evaluate the degree of mineralization. Four samples of each tooth were taken with the average being taken as the result. In each sample, the relative concentrations (wt %) of Ca, P, Cl, Na were measured and results represented as oxide percentages. The total mineral content was quantified from the measurements. For calibration of Ca, P, Cl, Na analysis the following standards were used: jadeite, P-apatite, tugtupite, magnesium oxide and wollastonite (Agar Scientific Limited, Stansted, United Kingdom), respectively. The EPMA operating conditions were 15 kV acceleration voltages, flowing speed of 15 nA and probe diameter of 5 μm.

Scanning electron microscopy (SEM) and energy dispersive spectrometry (EDS)

SEM analyses were performed with a Jeol JSM-6400 microscope (Jeol, Tokyo, Japan) as described previously (Koivukangas *et al.*, 2002). Back scatter electron

BSE images were used to evaluate the degree of mineralization. To distinguish between dentin and enamel, SEM images were obtained using BSE. In this method, the BSE signal is converted into a digital gray-scale image, where the intensity (gray level) of any pixel in the image is proportional to the mean atomic number of the corresponding location on the target material (Koivukangas *et al.*, 2002). BSE images were collected at 2664 x 2000 pixel resolution with 256 gray levels. To ensure the stability of the instruments, the BSE images were calibrated using a cobalt standard. One BSE image at 300 X magnification was collected from the cross section of the tooth crown and spectrums 1-3 were analyzed from different zones of enamel and spectrum 4 from dentin. The relative concentrations (wt %) of Ca, P, Cl, O, Na and the total mineral content in each sample were quantified using EDS (INCA 3.03; Oxford Instruments, Witney, UK). The SEM operating conditions were 15kV acceleration voltage with a working distance of 15mm.

4.10 Statistics

The data were analyzed using litter means (**I, II, III**). Weight of rats, mesio-distal length of the mandibular molars, length of molars, bone densitometry parameters, maximal breaking force and stiffness of the bones were evaluated with analysis of variance (ANOVA) followed by the least significant difference (LSD) test in the cases where the data displayed a normal distribution. If Levene's test showed $p<0.01$ even after appropriate transformations, nonparametric Kruskal-Wallis ANOVA combined with Mann-Whitney U was used. The limit of statistical significance was set at $p<0.05$. Fisher's Exact Test was used to test the proportion of erupted molars **(I)** and proportion of affected litters **(II)**.

In the caries study, the data were analyzed both including and excluding third molars **(IV)**. Due to similar outcome of the analyses, the data are presented including the third molars. There were no sex related differences in caries susceptibility and therefore the lesion results are shown collectively for females and males. Animal weight and total weight of consumed water and feed were tested using one-way analysis of variance followed by the least significant difference (LSD) test. If the variances between groups differed significantly, non-parametric Kruskal-Wallis combined with Mann-Whitney U was used. The proportions of animals with four third molar, caries lesions or T-lesions only were tested using Fisher's Exact Test.

5 RESULTS

5.1 Teeth as target organs of perinatal TCDD exposure

5.1.1 Arrested development of the third molars

The most dramatic effect of perinatal TCDD exposure was the total block of the third molar development **(I, II, IV)** (Fig 5, Table 2). This effect was seen in all of the three rat lines at maternal dose level 1 µg/kg **(I)**. However, line C rats were the most sensitive to this effect: 60% and 50 % of the male and female offspring, respectively, were affected (had at least one missing third molar) after maternal exposure on GD15 **(I)**. Mandibular third molars were more sensitive than maxillary third molars; proportion of missing maxillary molars was lower than that of mandibular molars (Fig 6) and in line C rats, maxillary molar agenesis was always combined with mandibular molar agenesis but not vice versa. In addition, in study **IV** 8% of line C offspring exposed to 0.1 µg/kg TCDD lacked third molars. In line A 5% and in line B 6% of studied female offspring had missing third molars after maternal exposure to 1 µg/kg on GD15 **(I)**. When pregnant line A rats were exposed to 3 µg/kg on GD11, 33% of the surviving offspring lacked one mandibular third molar on PND40 (a pilot study reported in study **III**).

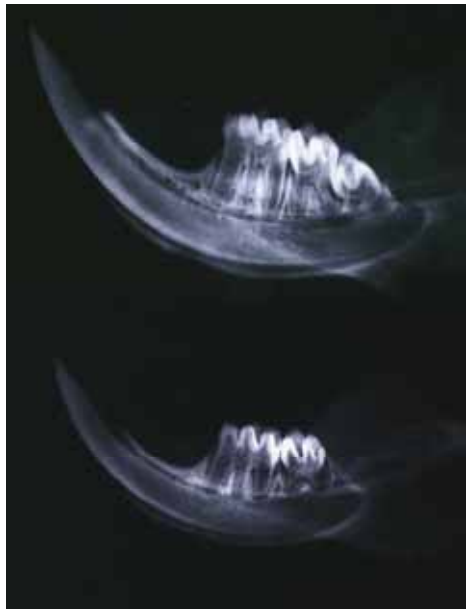

Figure 5. Mandibulas of line C rat offspring. A control animal (upper) has three molars, whereas an exposed offspring (lower) has only two molars.

The earlier the dosing occurred, the greater was the frequency of missing third molars **(II)** and the proportion of affected animals (Fig 6). Gestational exposure alone was sufficient to induce hypodontia, as seen in the cross-fostered group exposed only during gestation. Lactational exposure as such was insufficient to block the development of third molars; all of the offspring whose dam had only been exposed after delivery had all four third molars present. There was one exception to this rule: a cross-fostered offspring exposed only via lactation lacked one mandibular third molar. In this case, TCDD was present in the milk at the start of suckling. Even though the exposure started before the initiation of the first molars, it could not arrest the development of first or second molars.

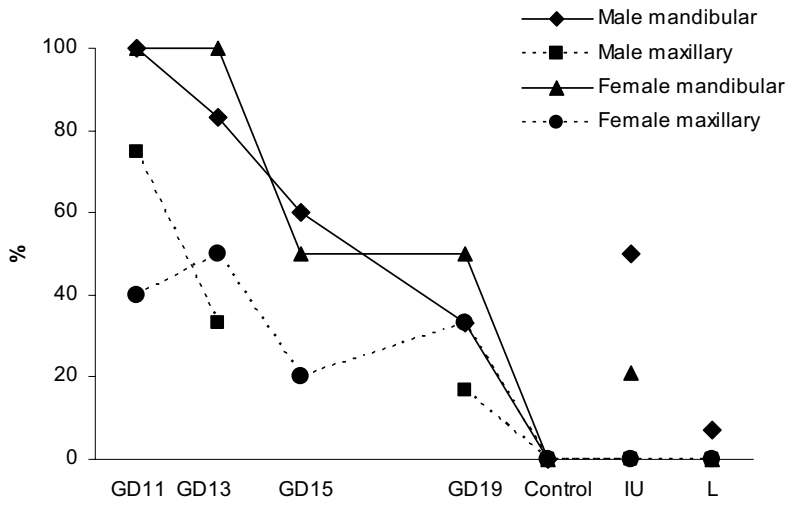

Figure 6.: Percentage of line C rat offspring exposed to TCDD at maternal dose of 1 μg/kg missing at least one mandibular and maxillary third molars in the time-course study **(II)**. IU: a group exposed only in utero between GD15-birth, L: a group exposed only via lactation

5.1.2 Eruption of third molars and incisors

TCDD exposure retarded the eruption of mandibular third molars dose-dependently (**I**: Fig 2). The effect was significant in line A rats on PND35 **(I)** and apparent, but not as clear in other lines due to differences in the eruption rate in control animals in different rat lines. On PND40 (**II**: Table 2) the eruption of third molars was retarded in line C rats this being mainly attributable to the mandibular third molars. The fact that the used maternal dose, 1 μg/kg, blocks third molar development at such a high frequency, interfered with the assessment of this effect.

The eruption of mandibular incisors was accelerated by TCDD exposure in line C rats (**II**: Fig5). This effect was time-dependent in a manner that earlier exposure accelerated eruption more than later exposure. TCDD exposure did not affect the eruption of maxillary incisors **(II)**.

Table 2: The percentages of animals missing at least one third molar in the different studies

Rat line	Maternal dose (µg/kg)	Dosing day	Sex	Mandibular 3rd molars [a)]	Maxillary 3rd molars [a)b)]	No. of animals studied
C [I+II]	0	GD15	♂	0	0 (17)	33
C [II, c)]	1	GD15[c)]	♂	50	0	8
C [II, d)]	1	PND0[d)]	♂	7	0	14
C [I+II]	0	GD15	♀	0	0 (29)	34
C [II, c)]	1	GD15[c)]	♀	21	0	14
C [II, d)]	1	PND0[d)]	♀	0	0	12
C [IV]	0.1	GD15	♂+♀	12	4	25
C [IV]	1	GD15	♂+♀	95	53	20
A [I]	0	GD15	♂	0	0 (5)	12
A [I]	1	GD15	♂	0	0 (3)	19
A [I]	0	GD15	♀	0	0 (13)	16
A [I]	1	GD15	♀	5	0 (11)	19
B [I]	0	GD15	♂	0	0 (2)	14
B [I]	1	GD15	♂	0	0 (2)	11
B [I]	0	GD15	♀	0	0 (8)	9
B [I]	1	GD15	♀	0	7 (14)	18

a) Percentage of animals missing target molars b) In parenthesis is the number of studied upper jaws in cases where not all upper jaws were studied c) a cross-fostered group where exposure took place only during gestation (GD15-birth) d) a cross-fostered group where exposure took place only via lactation (birth-weaning). Ns: not studied

5.1.3 Size of mandibular molars after TCDD exposure

TCDD exposure reduced the size of offspring mandibular molars. This was dependent a) on the dose (**I**: Fig 3 and Fig 4) and b) on the developmental stage of the molars (**II**: Fig 4). The mesio-distal length of the third mandibular molar was reduced to the greatest extent, and the first molars to the least. The genetic background did not affect sensitivity to this defect. In general, the molars and skull bones appeared to be less dense, i.e. less mineralized, in the radiographs even though this was not quantitatively measured.

5.1.4 TCDD exposure and caries susceptibility

Perinatal TCDD exposure increased the total number of caries lesions in sagittal hemisections in rats challenged with cariogenic treatment. The total number of caries lesions was significantly increased at the lowest maternal dose level of 0.03 µg/kg and it increased somewhat dose-dependently. The mean number of severe caries lesions involving dentin in the mandibular molars increased to 1.2 per animal at the highest maternal dose of 1 µg/kg as compared to 0.4 per animal in group C-2 or 0.5 per animal at maternal dose level of 0.03 µg/kg (**IV**, Fig 2).

5.1.5 Mineral composition

The weight percentage of calcium was dose-dependently diminished in the third molars, with the reduction being statistically significant in the 1 µg/kg dose group compared to controls **(IV)**. However, the dose-response was dissimilar to that seen for caries sensitivity. A very slight, though statistically significant, reduction was detected for magnesium oxide at the maternal dose level 0.3 µg/kg.

5.2 Bone defects only in line C after combined gestational and lactational TCDD exposure

5.2.1 Bone quality alterations only in line C

Gestational and lactational TCDD exposure affected the development of offspring bones only in line C rats **(III)**. The defects were detected at the highest maternal dose tested, 1 µg/kg, that caused some increased mortality in the offspring when dosed in the midpoint of pregnancy, on GD11 or GD13 **(II)**. TCDD exposure altered bone geometry, reduced bone mineral density and lowered the mechanical quality of

the bones. These changes were mainly reversible, because they were not detected at statistically significant levels in the exposed animals at the age of one year.

5.2.2 Geometrical changes

TCDD reduced the length of femur and tibia, though only at the dose 1 μg/kg (**III**, Table 2). Cross-sectional area of cortex (CSA), endosteal circumference (ENDO) and periosteal circumference were decreased at the same dose, though not significantly in all groups exposed at different times of gestation. (**III**, Table 2). The geometrical properties of bones from one year old rats were similar between exposed and control animals.

5.2.3 Bone mineral density

Bone mineral density (BMD) decreased in tibia and femur of TCDD exposed rats at maternal dose of 1 μg/kg. BMD was statistically significantly smaller only in animals exposed both in utero and via lactation (**III**, Table 2). BMD had normalised in the exposed rats by the age of one year.

5.2.4 Mechanical parameters

The mechanical strength of long bones, represented as breaking force and stiffness, was reduced in the rats exposed to a maternal dose of 1 μg/kg both during gestation and via lactation. (**III**, Table 3, Figure 3). At the age of one year, there were no significant reductions in the mechanical parameters. However, stiffness in both long bones and breaking force in femur were still 10% lower than those of the controls. On the contrary, femoral neck stiffness was increased at this age (**III**, Table 3).

6 DISCUSSION

6.1 Teeth

Developing teeth proved to be very sensitive to dioxin-induced developmental toxicity. In the present study, even the lowest maternal dose of 0.03 µg/kg TCDD significantly reduced the size of the molars in two rat lines. The defects ranged from deviated tooth eruption to agenesis of the third molar. The third molars were the most sensitive of the molars to TCDD-induced alterations in the development even when tested in a time-course study which included exposure before the initiation of the first molar. Agenesis of molars is a common anomaly affecting most often third molars. Up to 25% of the normal population lack one or more third molars (Thesleff, 1997). However, there were no cases of missing molars in control animals in this study.

6.1.1 Critical time of sensitivity for molar agenesis

The critical window of sensitivity is during pregnancy since lactational exposure alone at the dose levels used is not sufficient to interrupt third molar development. In all, 50-100% of line C rats exposed to 1 µg/kg TCDD at different times during pregnancy lacked one or more third mandibular molars. Only one animal exposed only via lactation lacked a third molar; this cross-fostered animal received TCDD in the milk from the start of lactation (i.e. the surrogate dam had been exposed on GD15). For other postnatally exposed groups, the dams were exposed after parturition. The third molars are initiated in rats on GD20 (Shellis and Berkovitz, 1981) and it appears that the most sensitive time for molar agenesis is initiation. The sensitivity to molar agenesis decreases rapidly after birth, because none of the offspring lactated by a post-delivery exposed dam lacked molars. It has been reported that a higher dose (50 µg/kg) administered to the dam is still able to arrest molar development on PND1 (Lukinmaa *et al.*, 2001). The fact that embryonic murine teeth *in vitro* proved to be most sensitive to TCDD before reaching early bud stage (Partanen *et al.*, 2004a) provides further support for the theory that initiation is the most sensitive stage. Interestingly, the frequency of missing molars increased if dams were exposed some days before third molar initiation as compared with those exposed just before third molar initiation **(II)**. This indicates that TCDD exposure affects the cascades preceding molar initiation and in this way makes oral tissues more sensitive to molar agenesis. In addition, lactational TCDD exposure increased the proportion of animals with missing molars. Nearly one in three (32%), of cross-fostered animals exposed *in utero* (between GD15 and birth) had missing third

molars but in a group with combined gestational exposure - starting on GD15 - and continuous lactational exposure, the proportion of animals with missing molars increased to 55%. AHR and ARNT, the mediators of TCDD toxicity, are expressed in embryonic murine teeth epithelium during the early stages of dental development and in secretory odontoblasts and ameloblasts, but not from the bud to the bell stage (Sahlberg *et al.*, 2002).

It appears that gestational exposure, even though very limited in amount as compared with lactational exposure (**III**, (Li *et al.*, 1995)), is sufficient to block tooth development across species. For example, maternal exposure on GD18 resulted in 0.07% of total dose per gram foetal liver on GD20, whereas the values after one or four days of lactation were 0.65% and 2.88%, respectively. *In utero* exposure has resulted in hypodontia in a rhesus monkey study, where a stillborn monkey was missing one tooth and one offspring that died on PND1 had incomplete calcification. Both monkey offspring exhibited perinatally erupted teeth, additionally a third offspring that died on PND26 had incomplete calcification, hypodontia and precocious eruption. In this study, maternal exposure had started with a loading dose of 0.03 or 0.3 µg/kg s.c. on GD20, approximately two weeks before the initiation of the first teeth. To achieve a steady body burden, a maintenance dose of 0.0015 or 0.015 µg/kg was given s.c. every 30 days during pregnancy and lactation until PND90. Dental alterations were detected only in the higher dose group. Continued exposure via lactation increased the frequency of offspring with dental alterations but only to a minimal degree. There was a high frequency of offspring with dental abnormalities i.e. 50% in stillbirths and early postnatal deaths and 64% in surviving offspring (Yasuda et al., 2005a). Therefore the critical time of sensitivity to molar agenesis seems to be during gestation, perhaps at the time of initiation – early bud stage also in primates. The methods used for revealing dental aberrations in the monkey study were intraoral photography and radiography, but no measurements of tooth size were reported. More detailed analyses of possible low dose level alterations will hopefully appear. Even though the most sensitive stage of dental development occurs *in utero*, later exposure via lactation seems to exacerbate defects that have been initiated during gestation. Later exposure reduces molar size both in rats **(I, II)** *in vivo* and embryonic murine teeth *in vitro* (Partanen *et al.*, 1998).

6.1.2 Sensitivity differences between rat lines, role of AHR

TCDD induced defects in dental development – decreased mesio-distal length, deviated eruption, third molar agenesis - in all three of the studied rat lines. The differences between the lines is attributable to the distribution of resistance alleles: line A rats carry the mutated AHR, line B rats possess the unknown resistance allele

B and line C rats have no resistance alleles. Apparently, the mechanism behind most, if not all, developmental dental toxicity is independent of the resistance alleles and the dental defects can be classified as type I endpoints. The only defect showing some dependence on resistance genes was the agenesis of the third molar which was seen mainly in the line C rats. In lines A and B, only one offspring per line were lacking a third molar. The dose-dependency, and thereby classification to type I or II endpoints, of molar agenesis cannot be resolved due to steep increase in mortality with increasing dose. However, maternal dose of 3 µg/kg on GD11 resulted in arrested third molar development in 33% of line A animals surviving to PND40. The AHR may mediate developmental dental defects, but the presence of the truncated transactivation domain in line A rats does not protect against dental defects.

6.1.3 Susceptibility to caries and mineral composition of enamel and dentin

TCDD increased caries susceptibility in rats exposed during the foetal period and via lactation **(IV)**. The effect was clear already at the lowest dose level tested, 0.03 µg/kg to dam. In rats, this dose level led to TCDD concentration (in fat) that is comparable to high-end concentration in Finnish breast milk (in fat) in the 1980s (Alaluusua *et al.*, 1996b; Hurst *et al.*, 2000). At the highest dose level, 1 µg/kg, the frequency of severe lesions increased. The mineral composition of enamel and dentin was only minimally altered below 1 µg/kg. At the highest dose level, the relative calcium concentration decreased slightly but statistically significantly **(IV)**. Alterations in the relative mineral concentrations failed to explain increased caries susceptibility. However, the used analyse methods do not detect changes in enamel crystal lattice at the nanostructure level. Alterations in the crystal lattice could make the enamel unstable and easier to dissolve, and these could be detected with high resolution high voltage electron microscopy.

Caries is an infectious disease that is influenced by dietary habits and oral hygiene. Humans are continuously exposed to a plethora of compounds *via* the diet, and therefore possible effects of dioxins are easily masked in epidemiological studies. In humans, exposure to PCDD/Fs at background levels has been found not to correlate with caries (Alaluusua *et al.*, 1996b). Caries studies among accidentally or occupationally exposed people have provided ambiguous results on the effect of dioxin-like compounds on caries. Occupationally PCB-exposed mothers self-reported carious teeth in their children at an increasing frequency in correlation with breast-feeding time, but no link between caries and children's blood PCB levels could be established in a medical examination (Hara, 1985). In Yucheng subjects, a very slight, though significant (χ^2-test), increase in caries was detected (Rogan *et al.*,

1988). It is possible that the Yucheng babies in Taiwan reported to have carious teeth had actually mineralization defects that were misclassified as caries. One rare opportunity to examine the effects of almost pure TCDD exposure in humans occurred with the tragically exposed subjects from Seveso, but not even in these subjects did caries correlate with TCDD exposure (Alaluusua *et al.*, 2004). Even though there is no unambiguous evidence that PCDD/Fs or PCBs increase the risk of caries in humans, our results demonstrate that TCDD does possess the ability to increase caries susceptibility in rats.

6.1.4 Possible mechanisms

The main target in TCDD-induced dental toxicity appears to be dental epithelium. During the critical time of sensitivity, i.e. between initiation and the bud stage **(II)**, the epithelium is responsible for dental development (Thesleff and Nieminen, 2001). TCDD is known to affect developing epithelium *in vitro* and *in vivo*. In rats, gestational and lactational exposure to TCDD impaired epithelial branching and differentiation in seminal vesicle (Hamm *et al.*, 2000). In female rats, a similar level of TCDD exposure decreased the primary branches, lessened epithelial elongation and depressed the number of alveolar buds and lateral branches in mammary glands. The mammary glands were most sensitive on GD15, when the mammary epithelial bud forms and begins its migration into the fat pad (Fenton *et al.*, 2002). Epidermal growth factor receptor (EGFR) signalling is required for TCDD-induced dental toxicity, at least at high *in vitro* concentrations (Partanen *et al.*, 1998). In an *in vitro* study with embryonic murine teeth, TCDD at micromolar concentration depolarized odontoblasts and ameloblasts of normal mice leading to failure of mineralization of dentin matrix and enamel matrix deposition did not occur. In mice without a functional EGFR, dental development was close to normal; only the cuspal contour was slightly altered (Partanen *et al.*, 1998). McNulty (McNulty, 1985) suggested that in adult rhesus macaques (*Macaca mulatta*) the main mechanism for the effects of chlorinated polyaromatic compounds would be reversion from a specialized epithelia to more generalised cell type characteristic to the embryonic layer from which the specialised cells originated. It is possible that in developing animals, TCDD interferes with epithelial cell differentiation - possibly by interfering with EGFR signalling - and thus alters branching morphogenesis and organ development.

In normal development, apoptosis is the mechanism for regulating cell number and organ shape. In normal tooth development, apoptosis can be detected in epithelial cells from the bud stage to late bell stage (Matalova *et al.*, 2004). The epithelial-mesenchymal interactions regulate apoptosis, because the number of apoptotic cells was lower at the epithelial-mesenchymal interface than elsewhere, but in isolated

epithelium and mesenchyme apoptosis was abundant throughout the tissue (Vaahtokari *et al.*, 1996). At a high concentration (1 μM), TCDD increased apoptosis in epithelial cells destined to undergo apoptosis in murine embryonic dental cells *in vitro*, but failed to trigger apoptosis in cells that do not normally undergo programmed cell death. Apoptosis was detected in the dental lamina of TCDD-treated first and second molars and in the inner dental epithelium in the first molar cups tips (Partanen *et al.*, 2004a). Vaahtokari *et al*. (1996) showed that epidermal growth factor (at one concentration) prevented apoptosis in mesenchyme, giving further support for the possible involvement of EGFR-signalling in TCDD-induced developmental dental toxicity.

The fact that the dental effects were the more serious the earlier the exposure started might also be due to toxicity to the progenitor cells. Other possible explanations involve enhanced apoptosis or disrupted migration of ectomesenchymal progenitor cells preceding jaw bone development and tooth initiation. In Msx knockout mice - that congenitally lack teeth and have small mandibles- the apoptosis of dental epithelium was increased and the proliferation of dental mesencyme disrupted as compared to wild-type controls (Han *et al.*, 2003).

TCDD is known to induce deviations in the vascular development in fish and birds e.g. by evoking edema (Peterson *et al.*, 1993). TCDD-induced blood clotting and deviations in blood flow are associated with malformations in bones and agenesis of teeth in rainbow trout and medaka fish (Hornung *et al.*, 1999; Kawamura and Yamashita, 2002). Therefore, a limited supply of oxygen and nutrients might explain the altered development of mineralized structures. However, Teraoka *et al.* (Teraoka *et al.*, 2002) showed that the initial effect of TCDD on zebrafish embryo jaw development occurred before there was any measurable effect on local circulation. Therefore, it appears that TCDD affects has direct effects on forming bone, but decreased blood supply may further worsen the abnormally initiated bone development.

Dietary factors influence tooth development, for example providing a restricted protein supply for rat dams resulted in smaller molars with an altered cuspal pattern and delayed eruption of the third molars in the offspring. Salivary excretion was also diminished in the offspring of similarly malnourished rat dams. The size of the third molars was diminished more than the size of other molars due to maternal protein-energy deprivation in rat offspring. In these studies, there was no evidence to link deviant tooth development with altered calcium metabolism or collagen formation (Anon., 1979). Many alterations in dental development caused by malnutrition resemble those found in our studies. In the present studies, animals were given food *ad libitum* after weaning, but it remains an open question whether the prenatal and lactational nutrient supply had been impoverished due to TCDD exposure. In mice,

gestational TCDD exposure to 3 or 6 µg/kg for four consecutive days disrupted in a dose-dependent manner the embryo-maternal vascular barrier in the placental labyrinth, evoking haemorrhage of embryonic blood into the maternal circulation (Khera, 1992). Alterations in the blood supply for the foetus could cause foetal ischemia and decrease nutritional status and in that way disturb embryonic development. Repeated TCDD exposure during pregnancy also severely affects the mammary glands of pregnant mice which probably results in a restricted milk supply for the offspring after birth (Vorderstrasse *et al.*, 2004). TCDD-exposed line C rats weighed slightly less than control offspring after birth throughout studies **I** and **II**, and exposed line B rats were lightweight through the first postnatal week in study **I**. However, the weight difference in our studies was not nearly as great as that caused by protein-energy deprivation, a condition which has been associated with deviated dental development (Anon., 1979) and even the highest dose did not decrease maternal food intake or maternal weight. Furthermore, the doses that affected tooth development in our studies are only a fraction of those that needed to disrupt placental vascular development or maternal mammary gland development. The effects of TCDD on developing teeth, even though they may be similar to those induced by protein-energy deprivation probably are not primarily caused by a restriction of the nutrient supply to the foetuses.

6.2 Bone

Developing bones proved to be less sensitive to TCDD than developing teeth. TCDD evoked deviations in bone quality only at the highest tested maternal dose and only in the most sensitive rat line C. TCDD exposure during pregnancy and *via* lactation resulted in deviated geometry, lower bone mineral density and altered mechanistic properties in the bones of the offspring. The defects were somewhat transient probably being repaired by remodelling, because no signs of disturbance were detected in the one-year-old offspring. However, femoral breaking force and stiffness were still less than 90% of the control value in the exposed offspring at the age of one year. Strength and stiffness are important mechanical properties of bone. Strength and stiffness of a bone are affected both by bone tissue properties and its cross-sectional geometry. Strength describes the load that is required to break the whole bone. Stiffness ensures that bones will retract to their original form after momentary deformation by externally applied forces (van der Meulen *et al.*, 2001). The polar cross-sectional moment of inertia (PMI) is derived from density measurements using pQCT, and it describes the torsional strength of bone.

6.2.1 Mechanisms and the role of AHR

A wealth of data suggests that dioxin-induced alterations in bone are mediated via AHR. Both AHR and ARNT are expressed in osteoblasts and very strongly in osteoclasts (Naruse *et al.*, 2002; Ilvesaro *et al.*, 2005a). Developing bone expresses AHR and ARNT during gestation in mice (Abbott and Probst, 1995), and TCDD-was reported to induce AHR expression in zebra fish but this effect preceded altered bone development (Mattingly *et al.*, 2001). Another AHR ligand, 3-methylcholanthrene has been shown to impair skeletal development. 3-methylcholanthrene inhibits proliferation and differentiation of osteoblasts *in vitro* and *in vivo*, for example it depresses the expression of osteoblastic differentiation marker osteocalcin (Naruse *et al.*, 2002). TCDD-induced alterations in fish bone development can be suppressed by AHR-antagonists alpha-naphthoflavone (αNF) and resveratrol. Pure AHR antagonist action can alter adult fish bone remodelling; continuous αNF exposure for two months has resulted in a lack of posterior fins in almost all fish. This points to a role for AHR in normal bone remodelling (Kawamura and Yamashita, 2002). *In vitro*, resveratrol reversed TCDD induced suppression in osteogenesis, for example as measured by alkaline phosphatase activity reduction (Singh *et al.*, 2000). H/W rats with the truncated transactivation domain of AHR responded to chronic TCDD treatment with altered bone quality, though at higher doses than the sensitive L-E rats (Jämsä *et al.*, 2001). Moreover, there is a difference in bone geometry in mice with a constitutively active AHR compared to wild type mice (Wejheden *et al.*, 2005). Collectively these data suggest that (hyper)activation of AHR is involved in the TCDD-induced defects on both developing bone and adult bones undergoing remodelling. However, the effect is probably due to interactions in signalling cascades, because the DNA sequences for both alkaline phosphatase and osteocalcin lack an AHR binding region.

The altered bone development can be speculated to be due to deviated function of either bone-forming osteoblasts, bone-resorbing osteoclasts or both cell types. Neither the number nor the activity of mature osteoclasts was affected by TCDD (Ilvesaro *et al.*, 2005a). However, in preliminary studies from our collaborators (Ilvesaro *et al.*, 2005b), the differentiation of osteoclasts *in vitro* was severely affected by TCDD. TCDD does not affect the proliferation of osteoblasts *in vitro*, but post-confluently, TCDD suppressed the formation of the multicellular nodules that are responsible for the bone tissue-like organization (Gierthy *et al.*, 1994). Furthermore, in another osteogenesis model, inhibition of osteogenesis by TCDD was detectable during the osteoblastic differentiation phase (Singh *et al.*, 2000). 3-methylcholanthrene, the AHR ligand, inhibited cell proliferation in two osteoblastic cell lines *in vitro* and *in vivo*. 3-Methylcholanthrene has restricted calcification of embryonic murine metacarpals (Naruse *et al.*, 2002). Therefore it is likely that

TCDD-induced bone defects are a combination of disturbed osteoblastic and osteoclastic function. We are currently carrying out *in vitro* tests with osteoblasts and osteoclasts to determine which stages of differentiation are affected by TCDD. This is being done by measuring the expression levels of certain markers of bone development, such as alkaline phosphatase.

In a similar manner to teeth, the developments of cartilage and bone are dependent on interactions between epithelium and mesenchyme. For example, correct epithelial-mesenchymal interactions are required for normal development of bird beak (Wedden, 1987; MacDonald *et al.*, 2004), and isolation of epithelium and mesenchyme leads to altered skull development (MacDonald *et al.*, 2004). TCDD and other dioxin-like chemicals have been reported to disturb the development of craniofacial structures in fish and rats. Rainbow trout sac fry, exposed to TCDD, exhibited underdeveloped jaws and anterior nasal structures near to hatching time (Hornung *et al.*, 1999). In rats, exposure to high doses of a PCB/organochlorine mixture during embryonic development resulted in visible facial malformations such as rounded skull and underdeveloped snout and lower jaw (Bowers *et al.*, 2004). In our studies (**I, II, III**) we also occasionally noted animals in the 1 µg/kg group with rounded skulls resembling those depicted by Bowers et al (2004). A failure of down-regulation of fibroblastic growth factor 8 results in a truncation of the upper beak in a chicken mutant strain and apparently in rounded skull form (MacDonald *et al.*, 2004). TCDD is known to alter growth factor expression levels in embryonic tissues (Abbott and Birnbaum, 1990; Bryant *et al.*, 1997; Abbott *et al.*, 1998), therefore disturbed growth factor signalling may be involved in thealtered bone development induced by TCDD.

Body weight is an important determinant of bone density and it impacts on bone turnover. The TCDD exposed offspring were lighter than the control rats, and the most severe alterations in bone quality were detected in the groups exposed on GD11 and GD13 and thus the bone effects correlated with low body weight. Similarly to teeth, malnutrition affects bone development. Rat offspring deprived of energy and proteins have smaller long bones and mandibles than control rats (DiOrio *et al.*, 1973; Nakamoto and Miller, 1979), though the reductions are similar to those for body weight. Because in study **IV** the bone effects were observed only at the highest dose-level of TCDD, it is possible that differences in body weight may partly contribute to the altered bone quality observed in TCDD exposed rat offspring.

The bone quality parameters were only changed in line C rats at the dose levels used here. However, chronic exposure of adult rats has resulted in altered bone quality both in the sensitive L-E rats and the resistant H/W rats (Jämsä *et al.*, 2001). Interestingly, untreated H/W rats have shorter and thinner femurs than L-E rats (Stern *et al.*, 2005) described in (Stern, 2005), but this may be due to some genetic

difference other than the AHR structure. Preliminary results show that long bones of transgenic mice with a constitutively active AHR (CA-AHR) differ from wild type (WT) mice bones. The bones of CA-AHR mice appear to be thicker than bones of WT mice (Wejheden *et al.*, 2005). Collectively these data suggest that AHR has a physiological role in maintaining bone homeostasis. Bone quality endpoints probably can be designated as type II defects, but due to the steep increase in offspring mortality, no meaningful dose-response for developmental bone defects could be determined in embryonic rats. Trabecular bone is metabolically more active than cortical bone and could theoretically be affected more quickly and possibly at lower TCDD dose levels than required to disturb cortical bone. Further studies with our collaborators have shown that also trabecular bone is sensitive to TCDD; the most sensitive individual bone parameter, CSA of femoral metaphysis, was decreased after a chronic exposure to a total dose 0.17 µg/kg in adult rats (Stern *et al.*, 2005) described in (Stern, 2005). We are currently studying how TCDD exposure during gestation can affect trabecular bone in rat offspring in order to determine if trabecular bone is altered at a lower maternal dose level than 1 µg/kg. Another interesting parameter to study would be the organic component of bone, mainly type I collagen, which provides bone tissue with its elasticity. Bearing in mind that TCDD exposure can alter mechanical quality of the bones, this may be due to changes in the structure and number of collagen fibers in bone.

Treatment with 3-methylcholantrene, an AHR agonist, has suppressed DNA synthesis of human umbilical vascular endothelial cells and arrested cells at the G0/G1 phase of the cell cycle. 3-Methylcholanthrene also inhibited cell adhesion and chemotactic migration of endothelial cells. Moreover, alpha-naphthoflavone (αNF), a TCDD antagonist, ameliorated the inhibition of DNA synthesis induced by 3-methylcholanthrene (Juan *et al.*, 2006). This suggests that the AHR has a role in the antiangiogenesis seen after of 3-methylcholanthrene in human umbilical vascular endothelial cells. It would be interesting to examine whether TCDD exposure can affect the bone vascular structure or the volume of circulating blood in the mandible veins.

6.2.2 Critical time of sensitivity

The developing bones were the more severely affected the earlier during gestation TCDD exposure was started **(III)**. This suggests that the critical window of sensitivity occurs during gestation, as the amount of TCDD that reaches the embryos *via* placenta is much less than that occurring with lactational exposure (Li *et al.*, 1995). However, gestational exposure without continued lactational exposure changed only bone geometry and did not alter the mechanical properties of the bone.

Probably the deviations in the bone quality in the group exposed only *in utero* were mainly rectified between birth and the sampling day (PND40).

Developmental studies have revealed that TCDD can affect cartilage, bone and tooth development in fish. TCDD targeted craniofacial structures morphology (Hornung *et al.*, 1999; Carvalho *et al.*, 2004), and caused severe dysmorphogenesis in craniofacial structures of zebrafish (Mattingly *et al.*, 2001), calcification in spinal cord and spines in medaka fish (Kawamura and Yamashita, 2002) and rib and vertebral development in rainbow trout and zebrafish (Hornung *et al.*, 1999; Mattingly *et al.*, 2001). Absence of teeth was reported in exposed sac frys of rainbow trout 47 days after fertilization (Hornung *et al.*, 1999). Collectively these data show that the developing bone is sensitive to TCDD also in fish species.

6.3 Implications for PCDD/F risk assessment

Before the present studies were conducted, the available studies suggested that exposure to PCBs and PCDD/Fs could alter dental development. Exceptionally high accidental exposure to a mixture of PCDFs and PCBs induced perinatal teeth and retarded eruption of permanent teeth in human infants exposed *in utero* (Rogan *et al.*, 1988; Hamada, 1996). A high dose of TCDD, 1000 µg/kg, to juvenile rats induced pulpal perforation and defective dentin formation in continuously growing incisors (Alaluusua *et al.*, 1993). An epidemiological study involving a relatively small number of subjects (total 102 children) indicated that the mineralization defects in the permanent first molars correlated with total lactational exposure to PCDD/Fs. In that study, the concentration of breast milk was measured and total exposure calculated using that concentration and the breast feeding time as determinants (Alaluusua *et al.*, 1996b). These subjects (born in 1987) were from the normal Finnish population with prevailing European background exposure, and this study was the first to suggest that molar development could be one of the most sensitive endpoints of PCDD/F toxicity in man. However, the biological plausibility of the role of PCDD/Fs in human mineralization defects was rather weak due to differences in levels and timing of exposure and in the development of target teeth between the experimental study and epidemiological studies.

In addition to rats and humans, species in which TCDD or PCBs have been reported to induce dental defects are fish (Hornung *et al.*, 1999), non-humane primates (McNulty, 1985) and mice (an *in vitro* study) (Partanen *et al.*, 1998). The present studies were specifically designed to obtain information about how low doses of TCDD can affect mineralized tissue development in rodents during gestation or after birth. The dose scale was similar to that used in studies which have examined reproductive defects in rodents (Mably *et al.*, 1992a; Mably *et al.*, 1992b; Mably *et*

al., 1992c; Gray and Ostby, 1995; Gray *et al.*, 1997a; Wolf *et al.*, 1999) and the lowest maternal dose level, 0.03 µg/kg, is likely to result in similar magnitude of maternal fat tissue concentration (Hurst *et al.*, 2000) to that was measured in Finnish breast milk in the 1980s (Alaluusua *et al.*, 1996b). Therefore the results from these studies confirm the view that PCDD/Fs can indeed dose-dependently affect dental development at the low doses relevant to risk assessment. The most striking defect is molar agenesis, and the low-dose defects include delayed molar eruption rate, diminished size and increased caries susceptibility. In humans, the defects associated with dioxin exposure are a) hypodontia, b) enamel opacity that is a qualitative defect, and c) enamel hypoplasia that is a quantitative defect. These last two parameters require monitoring by an experienced observer and they were not included in our analyses of rat teeth. However, it is evident from the radiographs that the molars were thinner, less dense and thus the enamel was probably hypomineralized. Over the course of the present studies, further evidence about the ability of TCDD to induce dental defects has been gained from studies carried out in other laboratories with minks (Render *et al.*, 2001) and non-humane primates (Yasuda et al., 2005a). In particular, the rhesus monkey study conducted by Yasuda and co-workers (Yasuda et al., 2005a) is of special interest because it proves that TCDD can affect dental development in primate species that resemble humans much better than rodents. In that study by Yasuda and coworkers, the lowest effective dose was 300 ng/kg (loading dose) to the pregnant female combined with a maintenance dose of 15 ng/kg every 30 days during gestation and lactation. Blood and milk concentration analyses will reveal very important data about the effective tissue concentrations. Collectively these studies indicate that developmental dental alterations induced by TCDD is a common developmental disturbance among vertebrate species, which makes it noteworthy and, when combined with our results, means that it should be considered as being one of the most sensitive endpoints of TCDD-induced toxicity.

Before the present studies, there was also some evidence that the hard tissue development of wild animals could be affected by organochlorines. Skull-bone loss in Baltic grey seals occurred at the time of severe organochlorine contamination (Bergman *et al.*, 1992). Similarly, developmental deformities in birds, by indirect evidence, and in birds of prey living in contaminated areas, were associated with the presence of organochlorines (Gilbertson *et al.*, 1991; Bowerman *et al.*, 1995). In experimental conditions, PCBs affected bird skeletal development (Hoffman *et al.*, 1996; Summer *et al.*, 1996). Our studies show that TCDD indeed affects cortical bone development and our preliminary findings suggest that trabecular bone is more sensitive to TCDD-induced developmental toxicity than cortical bone. Most bone defects reported have occurred in the craniofacial area and long bones (Gilbertson *et al.*, 1991; Alaluusua *et al.*, 1993; Hoffman *et al.*, 1996; Summer *et al.*, 1996;

Hornung *et al.*, 1999; Bowers *et al.*, 2004). Most probably, these are the malformations which are most striking and easiest to measure. We are currently scrutinizing whether other regions of the skeleton, e.g. vertebrae, are also affected. Dental defects were at least partly independent of the dioxin resistance alleles, but cortical bone defects were only detected in the most sensitive rat line C devoid of the resistance alleles. Before the ongoing studies on the sensitivity of trabecular bone are completed, it is unclear whether developmental skeletal defects can be included into the categories of most sensitive, generic endpoints.

Data from PCDD/F and PCB accidents from Seveso (Italy), Yucheng (Taiwan) and Yusho (Japan) show that human embryos and infants respond to high dioxin-like compound exposure with hypodontia or altered bone development (Rogan *et al.*, 1988; Hamada, 1996; Alaluusua *et al.*, 2004). Since it is apparent that PCDD/Fs and PCBs can interfere with human hard tissue development at high exposure levels, the main issue for risk assessment is to determine at what exposure levels human embryos and infants as well as wild-life animals would be at a risk of suffering developmental defects. In addition, the sensitivity of bone and dental development in comparison to other developmental defects has to be assessed. Due to differences in half-lives and exposure schemes in humans and experimental animals, lipid based tissue concentrations or body burdens are better dose metrics for comparison than daily intakes. Reliable data on human background tissue concentrations are available from a recent study where PCDD/F concentrations were measured in 337 subcutaneous fat tissue samples. In the Finnish general population, the fat tissue PCDD/F concentration ranged from 4.4 to 145.5 pg/g WHO-TEQ and this correlated with age, and the average being 33.4 pg/g WHO-TEQ (Tuomisto *et al.*, 2004). Further analyses of the same population revealed that when the three most important dioxin-like PCBs (DL-PCB), PCB 77, PCB126 and PCB169 were included, total WHO-TEQ values ranged from 4.6 to 197.8 pg/kg in adipose tissue (Tijet *et al.*, 2006), with the average being 43.3 pg/g adipose tissue (Tuomisto JT, personal communication).

In rats, a bolus dose of 0.05 µg TCDD/kg body weight to pregnant dams on GD15 results in maternal fat tissue concentration of 177 pg/g fat on GD21 (Hurst *et al.*, 2000) and in our study **III** exposure to 0.5 µg TCDD/kg body weight on GD 15 resulted in maternal adipose tissue concentration 2185 pg/g fat. Linear extrapolation of these data predicts a maternal adipose tissue concentration of 100-120 pg/g fat after exposure to 0.03 µg TCDD/kg body weight. This estimated maternal adipose tissue concentration which is sufficient to induce developmental dental defects in offspring, is similar to the highest values measured in the Finnish average population (PCDD/F 145.5 pg WHO-TEQ/g fat) and close to average of this population (33.4 pg/g fat WHO-TEQ for PCDD/Fs and 43.3 pg/g fat WHO-TEQ for

PCDD/F + three DL-PCB). Thus, one must conclude that the existing margin of safety is narrow. Mother's milk concentrations between 1992 and 1994 were 4.9-34.4 I-TEQ pg/g fat for PCDD/Fs and 2.4-32.8 pg/g fat for PCBs (Kiviranta *et al.*, 1999). Figure 7 depicts the relation between maternal lipid PCDD/F concentration and hard tissue deviations in human or rat offspring and human subjects lipid PCDD/F concentration in the general Finnish population. Human breast milk data was measured during 1987; measurements of Seveso subjects were made of plasma samples collected in 1976, the same year as the accident. As the levels of PCDD/Fs have fallen in human milk, representing a decrease in overall exposure (Kiviranta, 2005), today there is a greater gap between exposure of human breast-fed infants and the adipose tissue concentration associated with developmental dental defects than there was in the 1980s. Furthermore, in the Finnish study younger, fertile-age subjects, had lower fat tissue concentrations than older subjects (Tuomisto *et al.*, 2004). There are a few facts that favour an optimistic view for humans in respect to their sensitivity to PCDD/F induced developmental defects. First of all, humans are exposed continuously to low amounts of PCDD/Fs whereas most experiments such as the present studies are conducted with a bolus dose. After a bolus dose, the fraction of maternal body burden that is transferred to the foetus is much higher than after subchronic exposure (Aylward *et al.*, 2005). Furthermore, it is possible that human tissues are less sensitive to TCDD induced effects than rodent tissue. Human AHR can only be detected in high amounts using similar buffer solutions that are required for stabilizing AHR in tissues from nonresponsive mice strains, and the ligand-binding subunit of human AHR differs from that of responsive mice strains. Additionally, the affinity of TCDD for the human AHR is generally lower than its affinity for rodent AHRs (Okey *et al.*, 1994). However, it is crucial to continue the monitoring of environmental and mother's milk levels of these compounds in a preventive manner. It is important not to forget the virtually non-existent margin of safety in 1987, when these compounds may well have evoked mineralization defects in the normal Finnish population.

Recent PCDD/F risk assessments of the WHO (WHO, 1998) and the EU (EU, 2001) have used reproductive defects as the most sensitive endpoints of dioxin induced toxicity and have used the lowest observed adverse effect level (LOAEL) of 0.05 µg/kg based on reduced ejaculated sperm counts (Gray *et al.*, 1997a) reinforced with other sensitive effects, such as permanently reduced daily sperm production and cauda epididymal sperm reserve after a maternal dose of 0.064 µg/kg (Mably *et al.*, 1992a). In our studies, the LOAEL was slightly lower, 0.03 µg/kg **(I, IV)**. Therefore, the development of the teeth and the reproductive system seems to be equally sensitive to TCDD, and the present results are in fairly good agreement with the LOAEL value of 0.05 µg/kg as the basis of dioxin risk assessment, and indicate that at least this value is unrealistically low. Based on the WHO report: "Consultation on

assessment of the health risk of dioxins; re-evaluation of the tolerable daily intake (TDI): Executive summary" (WHO, 2000), the estimated daily intake (EDI) for humans of 14 pg/kg body weight is related to rodent body burden which can result in decreased sperm count in offspring. WHO recommends that the daily intake of dioxin and dioxin-like substances should not exceed 4 pg/kg body weight, however with the ultimate goal being an intake level less than 1 pg/kg body weight. If the ratio 0.03/0.05 [LOAEL from our study/LOAEL from (Gray *et al.*, 1997a)] is applied to the human EDI, the a daily exposure to 8 pg/kg could be enough to induce a maternal body burden sufficiently high to affect offspring dentition. Currently, the daily intake levels are below 2 pg TEQ/kg in the Finnish adult population (Kiviranta *et al.*, 2001), which should be low enough to protect future generations, even though the margin of safety is still very narrow.

Since dioxin induced developmental dental defects are sensitive, permanent and possess some clinical significance, they are possibly better biomarkers for dioxins as compared with temporary effects, such as somewhat decreased prostate weight and seminal vesicle weight. The defects that are induced during dental formation can not be repaired, because teeth – unlike bones - are not remodelled after they are formed. Human first permanent molars are mineralized during the two first years of life, that is, during a period when there is high exposure via breast milk. The present results also strongly support the use of developing teeth, especially the permanent first molar, as a biomarker for dioxin exposure as originally suggested by Alaluusua et al., 1999. However, it is possible that also other substances such as non-halogenated polycyclic aromatic hydrocarbons can affect dental development. Overall, this study stresses the importance of considering developmental defects being the as most important endpoints of dioxin induced toxicity from the risk assessment point of view. Furthermore, it provides experimental evidence that developmental dental defects can be considered as potential indicators of dioxin exposure.

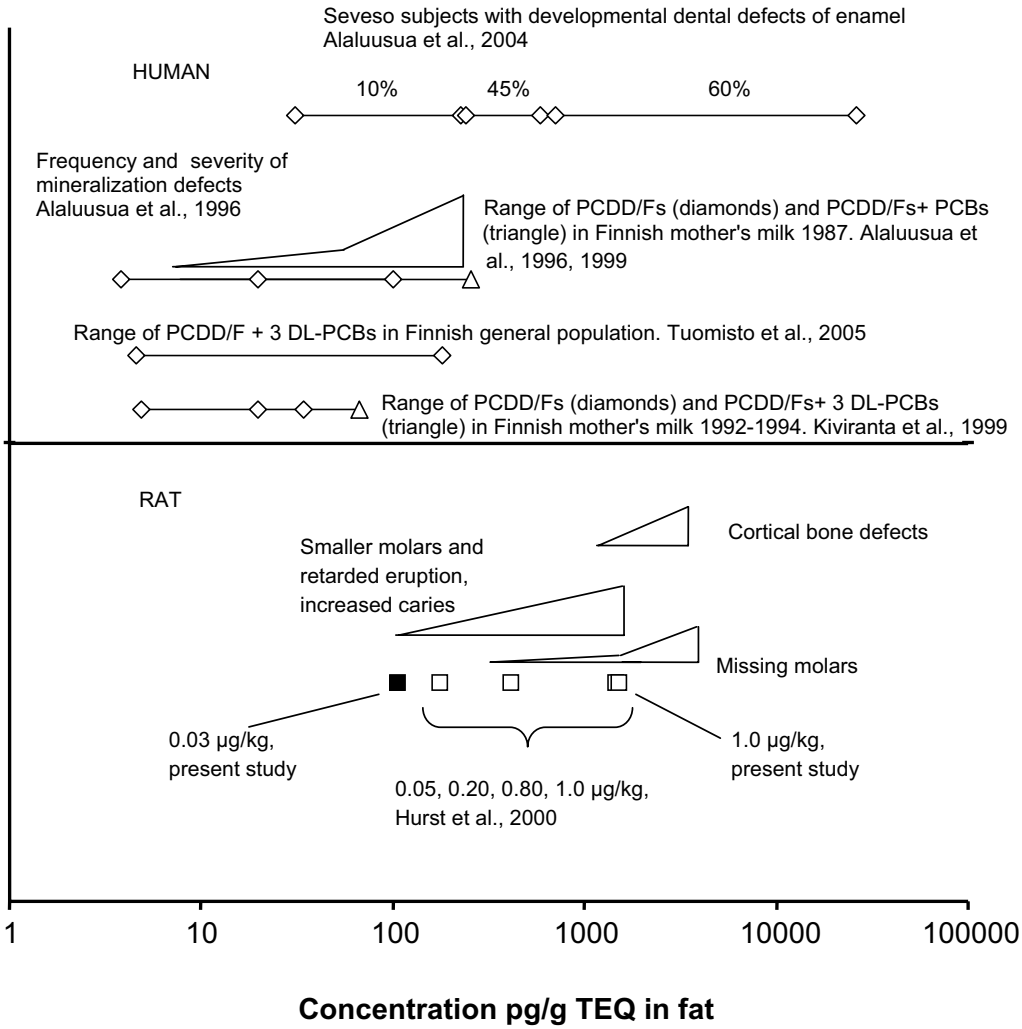

Figure 7 Maternal or subject fat based PCDD/F and PCB or dioxin-like (DL) PCB concentrations and deviations in hard tissue development in rat offspring and human subjects. Open diamonds (minimum, mean, and maximum of PCDD/Fs for Finnish studies) and triangles (maximum sum of PCDD/Fs and 3 DL-PCBs) denote measured concentrations in humans, open squares in rats. The lowest rat concentration (solid square) is a calculated value based on data from Hurst *et al*., 2000.

7 CONCLUSIONS

1. Developing rodent teeth are very sensitive to prenatal TCDD exposure. Low maternal TCDD doses alter molar eruption and decrease molar size.

2. The third molars are the most sensitive target teeth of TCDD induced developmental dental toxicity in the rat.

3. A maternal dose of 1 μg TCDD/kg body weight induces third molar agenesis in rat offspring.

4. Most developmental dental defects are not affected by the resistance alleles $Ahr^{hw/hw}$ and $B^{hw/hw}$.

5. Initiation is the most sensitive time for molar agenesis and therefore dental epithelium is believed to be the main target of TCDD-induced dental defects.

6. Perinatal TCDD exposure hampers cortical bone development at the same dose of TCDD which induces molar agenesis.

7. The sensitivity to cortical bone defects depends on the dioxin resistance alleles $Ahr^{hw/hw}$ and $B^{hw/hw}$.

8. TCDD induced cortical bone defects are mainly reversible.

9. Perinatal TCDD exposure increases caries susceptibility in rats. The effect is not explained by altered relative mineral amounts.

10. Third molar size is a sensitive biomarker for dioxin exposure in rats, and possibly in other animal species.

11. Body burden and background exposure to PCDD/Fs are currently less than that associated with adverse effects and suggested daily intake, respectively, in populations of fertile age, but the margin of safety is narrow.

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