Arch Toxicol (2013) 87:735–751
DOI 10.1007/s00204-012-0988-y

ORGAN TOXICITY AND MECHANISMS

Endocrine disruptors and bone metabolism

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Received: 28 July 2012 / Accepted: 21 November 2012 / Published online: 29 November 2012

Abstract Bone microenvironment is a complex dynamic equilibrium between osteoclasts and osteoblasts and is modulated by a wide variety of hormones and osteocyte mediators secreted in response to physiological and pathological conditions. The rate of remodeling involves tight coupling and regulation of both cells population and is regulated by a wide variety of hormones and mediators such as parathyroid hormone, prostaglandins, thyroid hormone, sex steroids, etc. It is also well documented that bone formation is easily influenced by the exposure of osteoblasts and osteoclasts to chemical compounds. Currently, humans and wildlife animals are exposed to various environmental xenoestrogens typically at low doses. These compounds, known as endocrine disruptor chemicals (EDCs), can alter the systemic hormonal regulation of the bone remodeling process and the skeletal formation. This review highlights the effects of the EDCs on mammalian bone turnover and development providing a macro and molecular view of their action.

Keywords Endocrine disruptors · Skeletal formation · Bone remodeling · Osteoblasts · Osteoclasts

Introduction

Bone remodeling is essential to skeletal-bone renewal and for calcium homeostasis. This process is under strict and complex regulation by multiple systemic hormonal and local acting factors such as the parathyroid hormone (PTH) (Neer et al. 2001; Hodsman et al. 2003; Sabbieti et al. 2009a), the bone morphogenetic proteins (BMPs) (Yamaguchi 1995; Rosen and Wozney 2002; Naganawa et al. 2008), the prostaglandins (PGs) (Agas et al. 2008, 2012; Sabbieti et al. 2008, 2010), etc. Bone and articular cartilage have also already been characterized as estrogen-responsive tissues (Spelsberg et al. 1999; Richmond et al. 2000). Emerging evidence indicates that exposure to environmental toxicants, known as EDCs, influences osteoblast and osteoclast-specific functions.

It is acknowledged that short-time exposure to estrogen during the first few days of life has positive effects on bone at adulthood (Migliaccio et al. 1996, 2000). These effects can depend on the duration of exposure during gestation as well as on the levels of estrogen (Masters et al. 2007). In those life stages, the prenatal and early postnatal periods have been shown to be the most vulnerable to endocrine disruption since organs and systems are changing more rapidly (Golub et al. 2004; Anway et al. 2005). Moreover, EDCs often presented complex dose–response curves because they act at different ranges of dosages from one system to another, with several reported effects on tissues; furthermore, they could involve cellular mechanisms by multiple signaling networks (Crews et al. 2000).

The molecular mechanisms standing on the EDCs action are complex and exerted upon multiple targets. An EDC may be an agonist at one hormone receptor but an antagonist at another one. Hormone availability is dependent on hormone biosynthesis, hormone transport to the target tissue, levels of hormone binding proteins, and hormone catabolism. EDCs have been described to interfere with all of these processes (You et al. 2001; Boas et al. 2006; Tabb and Blumberg 2006; Swedenborg et al. 2009).

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EDCs act as selected modulators of estrogen, androgen, thyroid, and other receptors (Schantz and Widholm 2001) by activating several signaling cascades, in particular those ones related to the aryl hydrocarbon receptor (AhR), a receptor involved in the metabolism of many xenobiotic substances, and to the nuclear receptors (NRs) (Petersen et al. 2006). Activation of AhR by EDCs can lead to increased degradation of steroid hormones as well as to higher estradiol production; meanwhile, altered NRs signaling can lead to further metabolic dysfunctions (Swedenborg et al. 2009). Although the specific mechanisms are still unknown, there are several indicators that explain the EDCs influence on bone structure by their disruptive effects in the signaling of specific key regulators of differentiation (e.g., Runx2 and osteocalcin) or the receptors downstream network. The disruption of the molecular mediators of bone formation and remodeling reflects to the bone strength, architecture, and density parameters such as the bone mineral density (BMD) and content (BMC), the bending force, the hardness, the plasticity of bones, etc. (Miettinen et al. 2005; Hermsen et al. 2008; Lind et al. 2009; Finnila et al. 2010; Rowas et al. 2012).

This review focuses on disrupting potency of various chemicals on bone macro and microenvironment in mammalians and underlines the time/dose effects of EDCs on skeletal development.

Disrupting chemicals

Organic tin compounds

Organotin compounds result from the addition of organic moieties to inorganic tin, which can bind until four hydrocarbon groups (mono-, di-, tri-, and tetra-substituted); they are known to be ubiquitous in the environment. Organotins have been widely used in agriculture and industry as biocides, wood preservatives, and stabilizers for polyvinylchloride polymers. Notably, tributyltin (TBT) and triphenyltin (TPT) have been widely used in antifouling paint for ships and fishing nets; they strongly contributed to contaminate marine areas. TBT and TPT are well-known as endocrine disruptors, and accumulation of these organotin compounds has been reported in marine fish and mammals (Kannan et al. 1996; Harino et al. 2000), resulting toxic toward a number of organs (Snoeij et al. 1987). Notably, TBT causes the main risk for humans exposed to these chemicals mainly via seafood in the diet (Risk and Policy Analysts limited 2005). Based on the immunological toxicity, a tolerable daily intake level of 0.25 μg/kg has been proposed (Penninks 1993). In this context, Adeeko et al. (2003) have investigated the consequences of exposure to TBT throughout gestation on pregnancy outcome using the Sprague–Dawley rat model. The authors stated that exposure to tributyltin chloride at the doses of 10 or 20 mg/kg, from 0 to 19 gestation days (GD), was associated with reduced ossification in the fetuses. In particular, at the concentration of 20 mg/kg, TBT was observed to cause misaligned sternebrae or sternoschisis with delayed ossification of the fetal pelvic girdle, skull, and limbs, while doses of 10 mg/kg reduced bone formation of the sternebrae. The above adverse effects of TBT on fetal skeletal ossification could be associated with the disturbances in maternal thyroid hormone homeostasis after administration of these chemicals (Adeeko et al. 2003). In this line, studies performed on mice demonstrated that injection of TBT (1 mg/kg) into pregnant dams inhibited the calcification of the supraoccipital bone in mouse fetuses; in addition, a delayed ossification in some metacarpals and metatarsals limbs was observed. In contrast, no considerable variations were reported on bone formation in the monobutyltin (MBT)-treated group (Tsukamoto et al. 2004). Furthermore, the molecular correspondence and the intracellular interactions of these in vivo data on TBT-bone effects were also investigated in vitro using, as model, rat calvarial osteoblastic cells (ROB cells). TBT (10^{-8} and 10^{-7} M) suppressed the expression levels of alkaline phosphatase (ALP) and osteocalcin (OCn) and it interfered with the calcium signaling and deposition in ROB cells. Thus, the delayed ossification of the fetal skeleton might be due to the TBT alteration of important differentiating markers and signaling cascades in osteoblasts (Tsukamoto et al. 2004). Regarding the effects of organotin compounds on osteoclasts metabolism, Yonezawa et al. (2007) reported that low concentrations (3–30 nM) of TBT and TPT can suppress osteoclast differentiation of the mouse monocytic RAW264.7 cells by decreasing nuclear factor of activated T cells (NFAT) c1 and by activating protein-1 (AP-1) expression via a retinoic acid receptor RAR/RXR-dependent pathway.

Data concerning the effects of TBT on tooth development and the dental hard tissue formation were provided by Salmela et al. (2008). The authors used mouse embryonic molar tooth cultures from fetuses at day 18 of gestation as a model; TBT administration (0.1, 0.5, and 1 μM) caused arrest of mineralization of dentin and enamel formation in the first molars which resulted smaller than the corresponding control teeth. In the second molars, TBT-exposed was observed a thinner predentin and cusps and fewer changes in the stellate reticulum. In addition, TBT was able to increase apoptosis in the epithelial enamel organ, mainly in the first molars. Thus, this organotin compound seems to be involved with the epithelial–mesenchymal interactions, essential for the tooth development. To note that the adverse effects of TBT were associated with the developmental stage of its administration (Salmela et al. 2008).

Indeed, has been reported that mouse mandibular first molars, cultured for 3, 5, or 7 days and exposed to 1.0 μM TBT, showed a decreased expression of genes involved in dentin and enamel mineralization such as OCn, matrix metalloproteinase 20, and dentin sialophosphoproteins. On the other hand, an increase of OCn was observed in the epithelial enamel organ with the inhibition of the dentin mineralization and enamel formation as potential consequences (Salmela et al. 2012).

Last but not least, it has to be noted that TBT regulates also human and mouse multipotent stem cells differentiation; namely, in vitro TBT leads to an increase in the number of human (doses 5 or 50 nM) and mouse (doses 50 nM) adipose-derived stromal stem cells, predisposed toward an adipocyte lineage, at the expense of the osteogenic lineage via PPARγ signaling (Kirchner et al. 2010). Additionally, in vivo studies from the same authors, concerning the effects of TBT on mouse stromal stem cells, supported the above in vitro findings. Specifically, pregnant dams were exposed to TBT (0.1 mg/kg) by gavage and, after 8 weeks, stromal stem cells of the offspring were isolated from the white epididymal/ovarian fat pads. The TBT-treated group exhibited a decreased osteogenic capacity of the stromal stem cells and increased adipogenic differentiation capacities (Kirchner et al. 2010). The above data on TBT-disrupted mesenchymal stem cells differentiation program were further supported from the recent investigation of Koskela et al. (2012). Bone marrow stromal cells (BMSCs) were isolated from femurs and tibias of male mice and treated with TBT (1 or 10 nM). High concentrations of TBT resulted in decreased ALP mRNA levels (culture day 6) and decreased, but not significantly, of OCn ones (culture day 10). Moreover, the authors claimed that the combined exposure to both TBT (10 nM) and a dioxin-like compound, TCDD (1 nM) (see below), results in synergistic adverse effects on osteoblasts precursors. The combined exposure affected molecular key role mediators of osteogenesis, such as ALP and OCn, and osteoclastogenesis much more than the individual exposures. Thus, it is understandable that the synergistic properties of these endocrine disruptors lead to impaired bone homeostasis (Koskela et al. 2012) throughout their harmful effects on mesenchymal stem cells (MSCs) differentiation. The skeletal targets of organotin compounds are schematized on Table 1.

Alkylphenols

Alkylphenol ethoxylates (APEs) are non-ionic surfactants, used in the manufacture of plastics, detergents, paints, and pesticides (Nimrod and Benson 1996). The major degradation products of APEs are 4-*tert*-octylphenol (OP) and 4-nonylphenol (NP) (Hernando et al. 2004). APEs are diffused in various environmental compartments, commonly found in river sediments and water, (Giger et al. 1984) and can exert estrogenic-like effects in a wide range of wildlife species (Kwack et al. 2002; Kannan et al. 2003). The potential toxic effects of APEs were studied in various tissues, particularly on liver, kidney, spleen, blood (Barlas and Aydogan 2009; Hsieh et al. 2009), and bones. In this regard, it have been demonstrated that alkylphenols, as endocrine disruptors, play a critical role in bone volume and homeostasis. Particularly, in vitro administration of NP and OP from 10^{-9} to 10^{-6} M in ROB cultures resulted in suppressed osteoclast formation without notable observations on proliferation, differentiation, and mineralization of osteoblasts population. Moreover, in vivo administration of NP and OP (0.1 mg/kg of body weight) to pregnant mice at 10, 12, and 14 days post-coitus (dpc) has revealed an accelerated ossification of the sternebrae and a dawdling metatarsals ossification of fetuses at 17.5 dpc (Hagiwara et al. 2008). It is understandable that APEs-inhibited osteoclast formation leads to skeletal disorders through slight or considerable alterations on ossification at sternebrae, metatarsals, metacarpals, and sopraoccipitals levels. Further in vivo studies, based on OP administration perinatally and postnatally to mice, have evidenced a reduction in bone growth in width. The pregnant mice were exposed to drinking water containing 1 or 10 μg/ml OP from gestational day 10 and during the lactation period, and the pups were also exposed after weaning. Both OP concentrations decreased the OCn levels in female offspring and, albeit long bones length was preserved, the diaphysis the periosteal and the endosteal circumferences of the cortical bone were significantly decreased after administration of low OP doses (1 μg/ml). The reduced growth observed on the periosteal surface in the cortical bone at the diaphysis could be due to the decreased ALP expression and, as a consequence, to the reduced periosteal osteoblasts deposition (Kamei et al. 2008). On the other hand, high OP doses did not modify the cortical bone area at the diaphysis, but decreased the trabecular bone area at the distal metaphysis, suggesting that OP provoke dose-dependent and site-specific changes in bone homeostasis. It is noteworthy that the adverse effects of OP fundamentally involved female offspring (Kamei et al. 2008).

The OP effects on osteoblasts differentiation was also investigated using the multipotent C3H10T1/2 cell line. Interestingly, treatment with high doses of alkylphenols, from 2.42×10^{-5} M to 7.27×10^{-5} M, decreased ALP and transforming growth factor β2 (TGFβ2) expression with the consequential block of the multipotent cells to differentiate into osteoblasts (Miyawaki et al. 2008). The same concentration (10^{-5} M) of OP was also used by Kwack et al. (2002) with adverse effects on human estrogen-sensitive MCF-7 cells. According to the above

Table 1 Effects of organotin compounds on cell and tissue targets

Disrupting chemical	Species	Molecular targets	Skeletal targets	Dose	Authors
Organic tin compounds	Rats/mice		Sternebrae Limbs Skull	10 and 20 mg/kg	Adeeko et al. (2003)
	Rats	ALP, Ocn, Ca^{++}} OBs	Metacarpal Metatarsal Supraocipital bone	1 mg/kg 10^{-8} and 10^{-7} M	Tsukamoto et al. (2004)
	Mice	NFATc1, AP-1} OCs		3–30 nM	Yonezawa et al. (2007)
	Mice		Dental hard tissue	0.1, 0.5 and 1 μM	Salmela et al. (2008)
	Humans/ mice	Stromal stem cells differentiation		0.1 mg/kg	Kirchner et al. (2010)
	Mice	ALP, Ocn} BMSC		10 nM	Koskela et al. (2012)
	Mice	OCn, MMP20} ABs		1.0 μM	Salmela et al. (2012)

OBs osteoblasts, *OCs* osteoclasts, *ABs* adamantoblasts, *ALP* alkaline phosphatase, *OCn* osteocalcin, *NFATc1* nuclear factor of activated T cells c1, *AP-1* activating protein-1, *MMP20* matrix metalloproteinase-20

findings, alkylphenols influenced bone architecture through the downregulation of critical factors involved in osteoblasts and osteoclasts differentiation. Recently, we performed extended studies regarding the capacity of these compounds to interfere with the proliferative/survival properties of 17-β estradiol (17-β E_2) in mouse primary calvarial osteoblasts (COBs); namely, treatment with NP 10^{-4} M caused a massive cell death in COBs; meanwhile, treatment with NP 10^{-6} and 10^{-5} M activated both the extrinsic and the intrinsic apoptotic pathway through a sequence of events that involved the increase of Bax/Bcl2 ratio, mitochondria destabilization, caspases 9, caspases 3, caspases 8, and Bid activation. No statistically significant effects were found after NP 10^{-7} M administration (Sabbieti et al. 2011). Taking into account that alkylphenols induce weak estrogenic effects by binding the estrogen receptors (ERs) (White et al. 1994), but are also considered potent anti-apoptotic agents on osteoblasts, we further investigated the ability of NP to interfere with 17-β E_2. Indeed, 10^{-7} M of 17-β E_2 increased ERα and ERβ expression in COBs, while exposure to 10^{-6} M of NP did not change the synthesis of these receptors. The concomitant treatment with NP (10^{-6} M) and 17-β E_2 (10^{-7} M) strongly suppressed the ERs up-regulation induced by 17-β E_2, indicating that NP is also able to compete with 17-β E_2 in regulating ERs. Considering that ERα or ERβ knock-out mice show decrease both in cortical and in cancellous bone mineral density (Windahl et al. 2002) and ERα is involved in osteoblasts proliferation and differentiation (Khalid et al. 2008; Chau et al. 2009), it is consequential that NP, by affecting estrogen receptors, could influence the molecular mediators involved in differentiation and survival processes in COBs (Sabbieti et al. 2011). In conclusion, these chemicals have the potential to affect the intracellular signaling processes, indispensable for bone remodeling and homeostasis, such as the gene expression regulated via the ERs and AR, the conversion of testosterone into estrogen by aromatase, and the function of AhR (Bonefeld-Jørgensen et al. 2007), involved in syntheses of steroids such as estrogens.

In humans, taking into account that the daily intake of NPs has been calculated to be ~7.5 μg (with intakes for breast-fed and bottle-fed infants of 0.2 and 1.4 μg/day, respectively) (Casajuana and Lacorte 2004), it is reasonable to speculate regarding the NP-adverse effects on bone tissue development in exposed children. The skeletal targets of APEs are schematized on Table 2.

Bisphenol A

Bisphenol A (4,4′-isopropylidenediphenol; BPA) is a class of synthetic monomers widely used in the production of polycarbonate plastic products and a constituent of epoxy and polystyrene resins extensively used in food-packaging industry and dentistry (Staples et al. 1998). BPA is considered as a xenoestrogen because it binds to estrogen receptors with approximately 10,000 times less affinity than E_2 and exhibits estrogenic properties when studied in in vitro assay systems (Kuiper et al. 1998). There are markedly different views regarding the potency of BPA, which varies in relation to E_2 as a function of both nuclear receptor subtypes, ERα or ERβ (Welshons et al. 2006). An interesting study, performed by Moors et al. (2006), revealed that BPA is readily transferred across the placenta of rat dams to the fetus. Recently, it has been reported that BPA and E_2 are generally equally potent as activators of cell membrane receptors and can stimulate rapid signaling cascades at concentrations as low as 0.01 pM (Watson et al. 2010).

Table 2 Effects of alkylphenols on cell and tissue targets

Disrupting chemical	Species	Molecular targets	Skeletal targets	Dose	Authors
Alkylphenols	Mice	OCs formation	Sternebrae	10^{-9} to 10^{-6} M	Hagiwara et al. (2008)
			Metatarsal	0.1 mg/kg	
			Metacarpal		
			Supraoccipital		
	Mice	OCn	Long bones	1 and 10 μg/ml	Kamei et al. (2008)
	Mice	ALP, TGFβ2} OBs		2.42×10^{-5} M to 7.27×10^{-5} M	Miyawaki et al. (2008)
	Mice	Bcl2/Bax, Bid, Caspases, ERα, ERβ} OBs		10^{-6} M	Sabbieti et al. (2011)

OBs osteoblasts, *OCs* osteoclasts, *ALP* alkaline phosphatase, *OCn* osteocalcin, *TGFβ2* transforming growth factor β2, *ER* estrogen receptor

The BPA effects on skeletal development was studied by Kim et al. (2001); they observed a treatment-related retardation in the ossification of fetal rats skeleton after gavage administration of the chemical to mated females from days 1 to 20 of gestation at high doses such as 100, 300, and 1,000 mg/kg/day (daily dose volume 10 ml/kg body weight). In particular, the number of ossification centers of sternebra, metacarpals, metatarsals, and phalanges was significantly decreased in the 1,000 mg/kg group, but no significant decrease was noticed in the 100 and 300 mg/kg groups. Other various types of skeletal variations, including enlarged fontanel, cervical rib, short supernumerary rib, short 13th rib, wavy rib, misshapen sternebra, bipartite ossification of sternebra, hemicentric thoracic centrum, bipartite ossification of thoracic centrum, dumbbell ossification of thoracic centrum, and incomplete ossification of pubis, were observed but there were no statistical differences either in the number of fetuses with skeletal variations or in the number of litters with affected fetuses among the groups (Kim et al. 2001). Albeit high BPA doses results toxic for the bone turnover and skeletal development, other studies evidenced that lower doses (0.1 and 1 % w/w) of this chemical in diet, for 5 months, prevent bone loss in female mice lacking the aromatase gene Cyp19. In particular, BPA-diet completely reversed the loss of femoral trabecular bone observed in Cyp19 knockout mice by increasing femoral BMD in a dose-dependent manner (1 % w/w BPA administration was more effective than 0.1 % w/w) and did not alter the femoral bone density in wild-type mice. Thus, BPA exert estrogenic activity without evident adverse effects (Toda et al. 2002). Recent studies, focused on femoral geometry and biomechanical strength, revealed that 10 μg/kg/day of BPA treatment in mice from gestation day 11 to postnatal day 12 increased femur length of 2.3 and 1.0 % in males and females. Moreover, exposure to BPA tended to decrease energy to failure by 10.3 % in females, but had no effect on energy to failure in males. Overall, BPA caused few significant alterations in bone strength. Indeed, in male mice BPA treatment increased femur length, but it had no effect on femur strength (Pelch et al. 2012).

Effects on human health from exposure to low doses of BPA are controversial. The body of evidences in BPA-influenced bone metabolism is less extensive than that into BPA's potential effects on reproductive hormones; hence, this appear to be an area for active investigation.

The skeletal targets of BPA are depicted on Table 3.

Diethylstilbestrol

In order to prevent miscarriages and to suppress postpartum lactation, during the 1950s and 1960s, diethylstilbestrol (DES) was a widely used estrogen agonist. It has been also used till late 1970s in agriculture, addicted into the feeding stuff as an ingredient to help growth (Greenberg and Robert 1982). Regarding the adverse effects, DES was identified as a transplacental carcinogen, causing unusual clear-cell vaginal carcinomas of the adolescent girls who had been prenatally exposed (Herbst and Scully 1970; Treffers et al. 2001). Albeit DES is no longer prescribed to pregnant women, it has been extensively used as a positive control to study the effects of developmental xenoestrogen exposure. Recent findings suggested that in utero, exposure to DES can induce epigenetic changes that affect the third generation (Titus-Ernstoff et al. 2010) which indicates the adverse transgenerational sequelae effects of this non-steroidal messenger.

In vivo studies, performed on male and female mice, showed that 4-week DES administrated at 500 μg/kg increased trabecular bone formation in the medullary cavity of the proximal metaphysis in femurs. Same results were observed in the sternum of all treated males at 500 μg/kg or higher, but not in treated females (McAnulty and Skydsgaard 2005). In addition, a sternum and femur

Table 3 Effects of bisphenol A on skeletal targets

Disrupting chemical	Species	Skeletal targets	Dose	Authors
Bisphenol A	Rats	Sternebrae	1,000 mg/kg	Kim et al. (2001)
		Metatarsal		
		Metacarpal		
		Phalanges		
		Ribs		
	Mice	Long bones	0.1 and 1 % w/w	Toda et al. (2002)
	Mice	Long bones	10 μg/kg	Pelch et al. (2012)

hyperostosis was observed in all animals treated with high doses of DES; moreover, fibro-osseous lesions (FOLs), characterized by accelerated osteoblastic bone turnover with concurrent fibroplasias, were found. Taking into account that osteosarcomas in mice arise from these areas of osteofibrosis and bony trabecular proliferation in the medullary cavity after long-term administration (360 days) of DES (Highman et al. 1981), the above findings suggested that FOLs can be considered preneoplastic lesions in mice; thus, 4-weeks-DES treatment exerted cancerogenic effects on bone.

Further studies claimed that female mice administrated with DES at 2 mg/kg/day (dissolved in corn oil and received with the diet) for 4 months showed increased femur and lumbar vertebrae (LV) 1–4 BMD and BMC, while decreased LV3 dimensions were observed. Divergent effects were observed in males treated mice, with decreased LV3 dimensions and no significant changes on LV1–4 BMD and BMC (Ward and Piekarz 2007). Furthermore, DES was administrated subcutaneously (2 mg/kg/day) in mice pups from postnatal day 1–5 in order to investigate the short-term neonatal effects of the utilized chemical. Mice were then killed after 4 months and, interestingly, positive results on bone architecture at adulthood were found: DES-treated females presented higher BMD of LV1 and LV3 with consequent stronger LV12, higher femur BMD and thus skeletal structure reinforcement. Differently, in male mice, a lower BMD and peak load of femur and lumbar vertebrae was observed, suggesting that DES exerted gender-specific outcomes on bone homeostasis (Kaludjerovic and Ward 2008).

While developmental exposure to high doses of DES resulted in positive effects on adult mice femoral and lumbar vertebrae geometry, recently findings by Pelch et al. (2012) have shown that low doses of DES had opposite effects on femoral geometry resulting in longer femurs in adulthood and negative effects on bone strength. Particularly, the authors performed femoral geometry and biomechanical strength analyses after DES administration (0.1 μg/kg/day) from gestation day 11 to postnatal day 12. DES exposure at low doses increased femur length at 2.0 % in females and tended to increase femur length at 1.3 % in males. Moreover, in males, DES administration increased cortical bone width at 12.0 %, decreased marrow cavity diameter at 12.9 %, and tended to decrease cortical bone width at 7.3 %. The authors argued that the decreased cortical bone width was associated with a decreased, 8.7 %, endosteal mediolateral diameter. However, no change in the periosteal mediolateral diameter was observed. The above findings suggest that, in DES-exposed males, changes in marrow diameter and cortical bone reflected in a change in geometry rather than a change in the amount of bone content. Additional exposure to DES decreased bone tensile strength in females and males by 8.2 and 18.3 %, respectively. Extended studies on whole bone biochemical properties of torsional ultimate strength, torsional stiffness, and energy to failure were also determined by torsional loading to failure. The combined effect of increased femur length and decreased material strength resulted in a trend toward decreased torsional ultimate strength. In DES-treated mice, it was evidenced a decreased energy to failure at 18.9 % in females and a tended to decrease energy to failure at 15.0 % in males (Pelch et al. 2012).

The overall ability for a bone to withstand torsional breaking is a combination of the geometry and the material property of the bone. An increase in length with a concurrent decrease in material strength tended to cause a decrease in ultimate torsional strength and, in due course, a trend toward decreased energy to failure. Thus, bones from developmentally exposed animals could not withstand as large of a torsional force prior to breaking as vehicle-exposed animals. The decreased tensile strength may be associated with increased cortical porosity and/or altered mineral composition after DES treatment. This suggests an overall negative impact of weaker bones that may be more likely to fracture (Pelch et al. 2012).

In a recent study, pregnant mice at 11–14 days of gestation and pups until 3 months of age were administrated with 0.1, 1.0, and 10 μg/kg/day of DES. Then, the BMD, BMC, bone area (BA), and trabecular bone area (TBA) were evaluated. DES-treated females (10 μg/kg/day) showed increased lumbar and femoral BA and TBA (lumbar 7 and 14 %; femoral 22 and 14 %, respectively) and BMC (18 %). Opposite results were obtained from male DES-treated mice; 0.1 and 1.0 μg/kg/day induced a decreased lumbar BA (2 %) and TBA (∼15 %), while 10 μg/kg/day caused a decreased femoral BA and TBA (10 %) and BMC (Rowas et al. 2012). Hence, these data indicate that DES increased BMC and TBA in adult females, but decreased the whole bone size in males.

Table 4 Effects of diethylstilbestrol on skeletal targets

Disrupting chemical	Species	Skeletal targets	Dose	Authors
Diethylstilbestrol	Non human primates	Long bones	0.5 mg/kg	Golub et al. (2003, 2004)
	Mice	Long bones Sternum	500 μg/kg	McAnulty and Skydsgaard (2005)
	Mice	Long bones Vertebrae	2 mg/kg	Ward and Piekarz (2007)
	Mice	Long bones Vertebrae	2 mg/kg	Kaludjerovic and Ward (2008)
	Mice	Long bones	0.1 μg/kg	Pelch et al. (2012)
	Mice	Long bones Vertebrae	0.1, 1.0 and 10 μg/kg	Rowas et al. (2012)

According to the authors, these findings reflect to more fragile bone in females and in feminized phenotype in males, since they present reduced bone size after DES administration.

DES effects were also investigated in female rhesus monkeys in the peripubertal period (6 months before to 6 months after the average of menarche; animals age: 24–36 months) exposed at 0.5 mg/kg/day. It was observed a decrease in long bone length (femur 6.7 %, tibia 4.7 %, humerus 5.8 % and radius 1.4 %), in BMC levels (lumbar spine, global proximal femur, and femur neck), and in BMD levels (femoral neck and global proximal femur) in DES-treated groups. It was also noticed that DES reduced the concentrations of calcium, phosphorous, and ALP (Golub et al. 2003, 2004). In synopsis, DES-exposed non-human primates presented a suppressed bone length development resulting in smaller bones with lower mineral mass and, as consequence, structural and morphological alterations. In light of the numerous data obtained in mammals, including the non-human primates, it is possible to hypothesize that DES could exert similarly adverse effects on human skeleton, although there is no specific information in this area. The overall targets of DES are reported on Table 4.

Dioxin and dioxin-like compounds

Dioxin and dioxin-like compounds are widespread pollutants, highly toxic and stable, considered immunotoxicants and enhancers of wasting syndrome, metabolic disturbance, cancer, reproductive toxicity, and developmental alteration in laboratory animals (Pohjanvirta and Tuomisto 1994). Humans are exposed to dioxins via food chain (Jones and de Voogt 1999). Since dioxins are chemicals with lipophilic properties, they can transfer from the adipose tissue to mother's milk and, consequently, to offspring at elevated concentrations (20–25 % of mother dioxin burden) (Tuomisto 2001). The estimated human daily intake of the polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and biphenyls (PCBs) currently is about 1–3 pg toxic equivalent quantity (TEQ) per kg body weight per day for adults (World Health Organization 1996). It has to be noted that the risks evaluation of daily intakes of dioxins and related compounds by humans and rats is complicated since the elimination half life is being about 7 years in man and about 20 days in rats (Pohjanvirta et al. 1990). The sensitivity of adult animals to many dioxin effects show a discrepancy among different species, albeit sensitivity to developmental defects seems to occur at quite similar doses in the majority of laboratory animals studied (Birnbaum 1995). The developmental defects were considered the most critical dioxin adverse effects from several years (World Health Organization 2000) and various studies were performed on teeth, bones, and reproductive system as significant targets of dioxin exposure.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is considered the most potent dioxin compound and its effects are mediated by an AhR which, upon exposure to TCDD, translocates into the nucleus, where it heterodimerizes with AhR nuclear translocator and binds to its specific DNA recognition sites to activate the transcription of dioxin responsive genes (Okey 2007). AhR is expressed in osteoblasts and in osteoclasts, with expression peaks observed after the matrix maturation stage and before the initiation of mineralization in differentiating osteoblasts (Ilvesaro et al. 2005; Ryan et al. 2007). It has been, also, demonstrated that AhR seems to play a physiological role in bone development, because AhR knock-out mice showed a lower incidence of large interfrontal bones than wild type in fetal stage (Peters et al. 1999).

Early in vitro studies performed by Gierthy et al. (1994) have revealed that 10^{-8} M of TCDD interferes with rat COBs metabolism, suppressing the post-confluent formation of multicellular nodules that develop during bone tissue-like organization. Furthermore, Singh et al. (2000), employing the chicken periosteal osteogenesis model and

the rat BMSCs, showed that 10^{-12} to 10^{-9} M TCDD administration over days 0–6 decreased collagen type I levels (30 %), ALP activity (33 %), and mineralization (75 % in BMSCs). Since the results are quite similar in both cell types, the authors suggested that TCDD exerted inhibitory effects on osteogenesis and indicated the AhR ligand as suppressors of osteo-differentiation; the authors concluded that this effect can be antagonized by the resveratrol, an AhR antagonist.

Other studies were performed on two rat strains, the Long-Evans (L-E) rat that is the most TCDD-sensitive animal strain, and the Han/Wistar (H/W) rat that is, on the other hand, the most resistant one to the acute lethality of TCDD (Jamsa et al. 2001). The H/W resistance was due to a point mutation of AhR resulting in an insertion/deletion type alternation at the 3′ end of the coding region of complementary DNA and in an altered transactivation domain (Pohjanvirta et al. 1998, 1999). Thus, H/W rats presented a smaller mutated AhR protein, albeit the binding affinity of TCDD to AhR resulted the same in both strains. Interestingly, 10-week-old rats were weekly treated by subcutaneous administration for 20 weeks with 0.17, 1.7, 17, and 170 μg/kg of TCDD (H/W only). In L-E rats, TCDD decreased tibial length at doses of 1.7 and 17 μg/kg; also a decrease of bone cross-sectional size, cortical area, and bone ash weight was observed. The effects of TCDD in H/W rats were slighter and only after 170 μg/kg of treatment. Diaphyseal geometry proved to be the most sensitive endpoint of toxicity after doses of 1.7 μg/kg TCDD for L-E and 17 μg/kg for H/W rats. Taken together the above findings suggested that TCDD, through AhR, affected bone modeling, mainly by reducing bone growth. In addition, the long-term adverse effect, exerted by the dioxin, seems to reflect greatly in a disturbance of regulatory pathways instead of an antiestrogenic action with consequent estrogenic deficiency (Jamsa et al. 2001). These reports, in line with Ryan et al. (2007), demonstrated that, in rat, inappropriate activation of the AhR by TCDD (5 and 10 nM) during osteoblast differentiation influenced gene expression and caused a significant reduction in ALP activity. Moreover, the authors stated that AhR transactivation by TCDD increased CYP1A1 and Cox-2 protein expression which results in an improper augmented bone formation and perturbed bone homeostasis. In point of fact, Korkalainen et al. (2009), employing BMSCs from mouse and rat tibia differentiating to osteoblasts and osteoclasts, claimed that TCDD (100 fM or 10 pM) decreased Runx-2, mRNA levels, ALP activity, and osteocalcin expression in vitro. An inhibition of osteoclast differentiation was observed, too. These adverse effects of the dioxin were abolished in AhR knock-out mice indicating that AhR signal plays a critical role on TCDD-disrupted osteodifferentiation. Additionally, TCDD (1 or 100 pM) exerted anti-estrogenic effects in the UMR-106 osteoblastic cell line by decreasing the Osteopontin (OPn) expression. The OPn down-regulation might be explained by direct regulation of the OPn gene throughout a cross-talk between the AhR signaling pathway and the ER signaling pathway, or both (Wejheden et al. 2006).

Other findings, concerning gestational and postnatal exposures to rats, revealed that dose- and time-dependent TCDD treatment resulted in adverse changes on bone geometry, mineral density, and mechanical properties. Interestingly, TCDD administration of 0.03, 0.1, 0.3, or 1 μg/kg in different time points from GD11 to PND19 evidenced a decreased tibias and femur length such as a decreased cross-sectional area of tibial and femoral cortex and a smaller endosteal and periosteal circumference in both long bones (dose 1 μg/kg). The same trend was observed for BMD of tibia and femur, for breaking force and stiffness of tibia, femur and femoral neck after 1 μg/kg TCDD treatment. It was underlined that the gestational exposure alone was not sufficient, but lactational exposure was required to provoke the bone defects (Miettinen et al. 2005). The authors claimed that most of the above effects were reversible (tibial and femoral length, BMD) at the age of 1 year. A further study on bone strength, architecture, and density after TCDD treatment was performed in rats by Finnila et al. (2010); namely, TCDD treatment (1 μg/kg), by a single intragastric dose on GD11, provoked decreased mineralization and altered bone geometry to the offspring at PND35 and PND70. In particular, reduced cortical BMC and BMD (max 16 and 0.9 %), tibial length, cross-sectional geometry, bending force of the tibia (max 20 %), and bone stiffness, as well as changes in hardness, plasticity index, and storage modulus, were observed in TCCD-treated group. Nanomechanical data suggested that TCDD-exposed offspring tibias were more ductile, softer, and less able to store energy than the control bone. Likewise, Lind et al. (2009) showed that short-term exposure to dioxin (50 μg/100 g for 5 days) in male rats provoked adverse effects in trabecular bone area and in bone mineral composition at tibial level. The short-term TCDD administration affected the whole bone cellular population, and the compositional changes could be due to osteoblastic death. Nishimura et al. (2009), in line with the above evidences, argued that one single oral dose of TCDD (15 μg/kg) at the mice dams provoked a marked increase in the amount of unmineralized osteoid as well as a dramatic reduction in mineralized bone in the proximal end of tibiae of the offspring on PND 14 and 21. The authors concluded that the characteristic toxic lesions caused by TCDD likely occur via suppression of osteoblastic bone formation, rather than promotion of osteoclastic bone resorption, which leads to the impairment of bone mineralization.

Reports of TCDD effects on primates were provided by Hermsen et al. (2008). The results of dioxin on bone

formation, composition, and geometry were in contrast from what observed previously on mouse and rat model. In particular, pregnant rhesus monkeys were treated with TCDD with a total dose of 40.5–42.0 or 405–420 ng/kg by repeated subcutaneous injections from GD 20 and followed every 30 days until 90 days after delivery. At the age of 7 years, femurs were dissected and an increased trabecular BMC, CSA, and periosteal circumference only in females were observed, suggesting bone formation through an estrogenic TCDD-effect. On the other hand, the anti-estrogenic effects of this compound provoked altered bone strength reflected in softer bone and eventual osteoporotic fractures in males.

3-Methylcholanthrene (3MC) is an aromatic hydrocarbon, as TCDD, that binds to the AhR and exerts immunotoxic and tumorigenic effects (Rier and Foster 2002; TenHave-Opbroek et al. 2000). Naruse et al. (2002) demonstrated that 3MC at 10^{-7} and 10^{-6} M decreased in vitro rat and mouse osteoblasts proliferation and differentiation, through signals carried by AhR. This dioxin-like compound affected bone formation by reducing ALP activity, osteocalcin and calcium deposition, thus disrupting indirectly the expression of critical genes involved in osteoblasts differentiation. Moreover, Naruse et al. (2004) claimed that this AhR ligand at 10^{-9} and 10^{-6} M inhibited the differentiation and fusion of osteoclasts, albeit their resorption activity was conserved in mouse clonal osteogenic stromal ST2 cells. Taken into account the in vitro dose–response of 3MC, it was affirmed that it inhibits osteoclastogenesis stronger than osteoblastogenesis. Further in vivo studies from the same authors revealed that injection of 1 mg/kg 3MC in pregnant mice caused adverse effects on fetuses bone modeling at 15.5 or 17.5 post-coitus days. A delay of ossification was observed in the sternebrae, limbs, cervical, thoracic, and lumbar vertebrae, and supraoccipital bone, but no changes were appreciated in femur, tibia, ulna, or radius (Naruse et al. 2002).

Benzo[α]pyrene (BaP) is a polycyclic aromatic hydrocarbon present in tobacco smoke and tar, shown to be implicated in the induction of cell proliferation as well as in tumors, including the osteosarcoma (Culp et al. 2000; Jeffy et al. 2002). In vitro studies performed by Tsai et al. (2004) demonstrated that BaP (1–10 μM) was able to increase cell proliferation in cultured rat osteoblasts and human osteosarcoma MG-63 cells. Moreover, the authors evidenced that BaP triggers cyclooxygenase-2 (COX-2) through the ER-related ERK-MAPK, and PI3K/Akt signaling pathways, independently. Regarding the BaP effects on osteoclast population, Voronov et al. (2005) stated that this dioxin-like compound (10^{-6} and 10^{-5} M) inhibited osteoclastogenesis and bone resorption in mouse macrophage cell line RAW264.7 and in dispersed rabbit osteoclasts only at high cell density. Furthermore, they affirmed that the BaP-suppressed osteoclasts effects are direct, via AhR–NF-κB competition, as well as indirect, via the stromal cells (Voronov et al. 2005, 2008). Moreover, BaP (1, 5, 10 μM) inhibits chondrogenesis and accelerates chondrocyte differentiation in E11/E12 stage of limb mouse bud BMSCs. The influence of BaP is AhR-dependent and may be due to BaP bio-activation leading to DNA damage, as a consequence of the crosstalk between AhR signaling and other pathways that regulate chondrocyte differentiation (Kung et al. 2012). Summarizing, it is well understood that BaP disrupts bone architecture affecting all cells population and leading to impaired bone remodeling.

Polychlorinated biphenyls (PCBs) are a group of halogenated aromatic hydrocarbon compounds widely used as diluents, flame retardants, fluids for capacitors and transformers; they are considered ubiquitous environmental contaminants (Hansen 1999). There are 209 different PCB congeners differing in the position of chlorine atoms and the degree of chlorination. These differences affect their physicochemical properties and biological activities. The non-ortho-substituted, coplanar congener 3,3′,4,4′,5-Pentachlorobiphenyl (PCB 126) is considered to be the most toxic PCB congener (Safe 1990) and shows high affinity to the AhR and high acute toxicity. Several findings suggested that PCB 126 might have estrogenic or anti-estrogenic properties depending on the estrogen status of the individual. A study performed in humans by Den Hond et al. (2002) evidenced a delayed sexual maturation in adolescents living in areas contaminated by PCBs and dioxins. The findings suggest that, in agreement with the concept of endocrine disruption, these xenoestrogens may impair male and female pubertal maturation. Moreover, PCBs may act in humans not only by decreasing the hormonal secretions, but also through direct interference with the androgen and estrogen receptors (Den Hond et al. 2002).

Actually, experimental studies of PCBs on ovariectomized or intact rats showed that these compounds (administrated for 3 months by intraperitoneally injections for a total dose 384 mg/kg) exerted structural and functional changes in rat bone tissue (Lind et al. 1999, 2000). As result, in estrogen-deprived tissues, PCB 126 exerted weak estrogenic activities indicated by the decrease in tibiae length and the increase of bone mineral density. On the other hand, in estrogen-rich tissues, PCB 126 exhibits antiestrogenic behavior reflecting in impaired bone mineralization of tibiae as indicated by the significant increases in organic content and in osteoid surface (Lind et al. 1999). In addition, functional analyses of long bones revealed an impaired bone strength, and biochemical analyses of the bone tissue proved that the changes in bone strength might be due to a substantial decrease in the collagen content in rats treated with PCB 126;namely, the polar moment of inertia of humeri from PCB126-exposed rats was about

15 % lower compared with controls (Lind et al. 2000). Further studies performed by Alvarez-Lloret et al. (2009) demonstrated that 3 months female rats exposed to 64 μg/kg of PCB126, (total dose 384 μg/kg), significantly altered the vertebral bone mineral composition at the molecular level. Specifically, the mineral composition in the treated group showed a lower relative degree of mineralization (−8.5 %) respect to the controls. Moreover, a significant increase in the trabecular density of vertebrae in exposed rats (+12 %) was observed. The authors speculated that this disturbance of bone mineralization was related to a reduction in thyroid hormone and vitamin D levels in serum, which regulation are critical for growth, differentiation, and regulation of bone tissue. In this view, it's well understood that PCB-induced disorders in vitamin D and, consequently, on bone mineral metabolism through a stimulation of bone resorption and inhibition of bone formation mechanism.

Moreover, it should be taken into account that, in humans, PCBs can bioaccumulate in the serum of the adolescents (1.67 nmol/L in boys and 1.02 nmol/L in girls) in polluted regions (Nawrot et al. 2002). In this context, it is possible to hypothesize that PBCs could exert impaired hormone production with potential effects on skeletal formation and development. In Table 5, the targets of dioxin and dioxin-like compounds on bone macro and microenvironment are reported.

Phthalate esters

Phthalate esters are considered global contaminants widely distributed in the environment. Since they are characterized by a moderate resistance to degradation, they often are present, at low levels, in food (Group 1986; Sharman et al. 1994). Phthalate toxic potential was referred to many years ago (Mayer et al. 1972), and it has been found that they can also be regarded as endocrine disruptors with estrogenic activity (Jobling et al. 1995; Harris et al. 1997). These chemicals have been found to cause a significant increase in the number of skeletal malformations as deformity of the thoracic vertebrae and fusion of the vertebral arches in rat fetuses (Ema et al. 1993). Also, teratogenicity and embryolethality was observed in 11–20 day rat fetuses of dams fed with a diet containing 2 % of the phthalate ester benzyl butyl phthalate (BBP); the administration of BBP during the first half of pregnancy produced embryolethality (Ema et al. 1992). In vitro investigations, performed to study the effects of BBP and di-*n*-butyl phthalate (DBP) in rat osteoblasts Py1a, evidenced that the above estrogen-mimicking compounds modified the intracellular localization of the fibroblast growth factor-2 (FGF-2), one of the most important regulators of bone remodeling (Hurley et al. 1994, 1998; Naganawa et al. 2006). In particular, 10^{-9} to10^{-6} M of BBP and DBP induced FGF-2 perinuclear accumulation and subsequent translocation into the nucleus (Menghi et al. 2001). The nuclear trafficking of FGF-2 (Sabbieti et al. 2005; Marchetti et al. 2006) plays important roles on nuclear events such as transcription in different ways; indeed, gene transcription can be inhibited, unchanged, or enhanced. Considered the above findings, it should be claimed the possible role of BBP and DBP during differentiation and bone formation, suggesting that the embryotoxicity and skeletal malformations in rats (Ema et al. 1992, 1993) could be attributed to the interference of phthalates with the intracellular signaling and functions of FGF-2. Accordingly, it was observed that pregnant rats after DBP administration by gastric intubation at dose of 750, 1,000, or 1,500 mg/kg/day on gestation days 7–9, 10–12, or 13–15 manifested a 100 % post-implantation loss in the 1,500 mg/kg/day dose group, an increase in the number of skeletal malformations (deformity of vertebral column) when treated with 750 and 1,000 mg DBP/kg/day on gestation days 7–9, and a dose-dependent increase in the number of external and internal malformations such as cleft palate and fusion of the sternebrae when exposed on gestation days 13–15 (Ema et al. 1994). Moreover, since microfilaments are crucial for the maintenance of cell shape and an altered assembly of actin fibers can be responsible for the perturbation of focal contacts and compromise the correct adhesion of osteocytes (Tanaka-Kamioka et al. 1998), the effects of BBP and DBP on actin cytoskeleton in Py1a cells were investigated. We demonstrated that after phthalate stimulation (10^{-6} M for 2 h or 10^{-3} M for 1 h) these endocrine disruptors act rapidly, transiently, and in a dose- and time-related manner on cell morphology by disrupting actin filaments; however, the initial conditions were restored after removal of the effectors (Marchetti et al. 2002). Studies to elucidate the transient effects evidenced that the actin cytoskeletal re-established conditions are dependent on new actin expression and synthesis (Agas et al. 2007). The actin microfilament disruption is reported to be of importance as a potentiator of cell growth (Gordon 2002). Indeed, we observed that BBP treatment increased rat osteoblasts proliferation, in parallel to an increased cyclin D3 expression, which specifically requires extracellular mitogenic stimuli for its activation (Agas et al. 2007). Since it was reported that the rat osteoblasts proliferation, induced by 17-β E_2, involves the increased expression of cyclin D3 (Fujita et al. 2002), we hypothesized that BBP mimics the 17-β E_2 effects on rat osteoblast as also previously stated by Zacharewski et al. (1998) and Yu et al. (2003). Moreover, a latest study performed by Bhat et al. (2012) showed that treatment with 10 μM of di 2-ethyl hexyl phthalate (DEHP) for 48 h increased rat calvarial osteoblast proliferation, while doses of 10 and 100 μM of this chemical decreased ALP, TAZ, Runx2, and collagen syntheses.

With reference to the in vitro studies on mouse model, we also demonstrated that both BBP and DBP at 10^{-6} M

Table 5 Effects of dioxin and dioxin-like compounds on cell and tissue targets

Disrupting chemicals	Species	Molecular targets	Skeletal targets	Dose	Authors
Dioxin and dioxin-like compounds					
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	Rats	Multicellular nodules} OBs		10 nM	Gierthy et al. (1994)
	Rats	Coll I, ALP, AhR		10^{-12} to 10^{-9} M	Singh et al. (2000)
	Rats	AhR	Long bones	1.7 and 17 mg/kg	Jamsa et al. (2001)
	Rats		Long bones	1 mg/kg	Miettinen et al. (2005)
	Rats	AhR, ALP, CYP1A1, Cox-2		5 and10 nM	Ryan et al. (2007)
	Non human primates		Long bones	40.5–42.0 and 405–420 ng/kg	Hermsen et al. (2008)
	Rats/mice	Runx-2, ALP, OCn, AhR		100 fM and 10 pM	Korkalainen et al. (2009)
	Rats		Long bones	50 mg	Lind et al. (2009)
	Mice		Long bones	15 mg/kg	Nishimura et al. (2009)
	Rats		Long bones	1 mg/kg	Finnila et al. (2010)
3-Methylcholanthrene	Rats/mice	AhR, ALP, OCn	Sternebrae	10^{-7} and 10^{-6} M	Naruse et al. (2002, 2004)
			Limbs	1 mg/kg	
			Vertebrae		
Benzo[a]pyrene	Rats/humans	ER, ERK-MAPK, PI3K/Akt} OBs		1–10 mM	Tsai et al. (2004)
	Mice/rabbits	AhR, NF-kB} OCs		10^{-6} and 10^{-5} M	Voronov et al. (2005, 2008)
	Mice	DNA damage, AhR} Chs		1, 5 and 10 mM	Kung et al. (2012)
Polychlorinated biphenyls	Rats		Long bones	64 mg/kg	Lind et al. (1999, 2000)
	Rats	Vitamin D	Vertebrae	64 mg/kg	Alvarez-Lloret et al. (2009)

OBs osteoblasts, *OCs* osteoclasts, *Chs* chondroblasts, *ALP* alkaline phosphatase, *OCn* osteocalcin, *ER* estrogen receptor, *AhR* aryl hydrocarbon receptor, *Coll I* collagen type I, *ERK* extracellular signal-related kinases, *MAPK* mitogen-activated protein kinases, *PI3K* phosphoinositide 3-kinase, *NF-kB* nuclear factor kappa-light-chain-enhancer of activated B cells

provoke an increase of DNA damage and a related phosphorylation of specific check points molecules as phospho-ATM (ser-1981) and phospho-p53 (ser-15 and ser-20) in MC3T3-E1 osteoblasts and mouse primary calvarial osteoblasts. Furthermore, we determined the effects of phthalate administration on critical apoptotic regulators and we found a decrease of mitochondrial potential, the cytochrome c release, and caspases activation (Sabbieti et al. 2009b). Interestingly, we showed that daily osteoblast treatment with 10^{-6} M BBP or DBP amplified the up-regulation of all the apoptotic markers indicating that phthalates exert cumulative effects with potential bone homeostasis perturbance. In the same study, we suggested that different models have dissimilar responses to EDCs, depending on the expression of cell cycle key regulator protein, p53. Indeed, both in mouse osteoblasts knocked-down for the p53 and in rat Py1a osteoblasts (that do not undergo phospho-p53 alteration by phthalates), BBP and DBP induced cell proliferation, instead of apoptosis, consistent with increase of c-myc and cyclins (Sabbieti et al. 2009b). In this context, an important question is to elucidate the functional meaning of increased p53 that was only found in treated mouse osteoblasts. This is a prerequisite for understanding the mechanism by which phthalates regulate the fate of the cells.

An interesting in vivo study shows that female rats, treated by oral gavage with di-isoheptyl phthalate (DIHP) (300 and 750 mg/kg) on gestational days 6–20, manifested numerous skeletal variations and malformations on fetuses including both rib and vertebral anomalies in DIHP high dose treated group (McKee et al. 2006). Similar results were obtained by Saillenfait et al. (2009) which studied the effects of di-*n*-hexyl phthalate (DnHP) and dicyclohexyl phthalate (DCHP) in female rats administered with DnHP

Table 6 Effects of phthalate esters on bones and osteoblasts (OBs) and osteoclasts (OCs) molecular targets

Disrupting chemical	Species	Molecular targets	Skeletal targets	Doses/treatments	Authors
Phthalate esters	Rats		Skeletal malformations	2 % w/w	Ema et al. (1992, 1993, 1994)
			Vertebrae	750 and 1,000 mg/kg	
	Rats	FGF-2		10^{-9} and 10^{-6} M	Menghi et al. (2001)
	Rats	Actin cytoskeleton		10^{-6} and 10^{-3} M	Marchetti et al. (2002)
			Ribs	300 and 750 mg/kg	McKee et al. (2006)
			Vertebrae		
	Rats	Lamin A, nuclear actin, cyclin D3} Obs		10^{-6} M	Agas et al. (2007)
	Mice	DNA damage, cytochrome C, Caspases, cyclins, p53} OBs		10^{-6} M	Sabbieti et al. (2009b)
	Rats		Ribs	0.25, 0.50 and 1 g/kg	Saillenfait et al. (2009, 2011)
	Rats	TAZ, Runx-2, collagen} OBs		10 and 100 μM	Bhat et al. (2012)

or DCHP at doses of 0, 250, 500, and 750 mg/kg/day, by gavage, on gestational days 6–20. The skeletal examination of fetuses revealed increased incidences of several variations: sternebral anomalies and cervical ribs were significantly elevated at 500 and 750 mg/kg/day and poorly ossified hyoid at 750 mg/kg/day. The incidence of 14th supernumerary ribs (mostly short) was also significantly greater than control at all doses and showed dose–response dependency (19, 61, 91, and 96 % of the fetuses at 0, 250, 500, and 750 mg/kg/day, respectively). Significant delayed ossification was noted in the hindlimb proximal phalanges at 250 mg/kg/day and higher doses and in the forelimb phalanges at 500 and 750 mg/kg/day. Further researches, by the same authors, aimed to investigate the di-*n*-heptyl phthalate (DHPP) and di-*n*-octyl (DnOP) phthalate effects in rat development. Dams were administered with 0, 0.25, 0.50, or 1 g/kg/day of DHPP or DnOP, by gavage, on gestation days 6–20. Skeletal examination of fetuses presented supernumerary lumbar ribs (more than 98 % short) at all dose levels. They were seen in 9, 43, 49, and 86 % of the fetuses at 0, 0.25, 0.50, and 1 g/kg/day and all fetuses had 26 presacral vertebrae. Moreover, the number of forelimb proximal phalanges was significantly lower than control at 1 g/kg/day, as was the number of hindlimb proximal phalanges at 0.5 and 1 g/kg/day (Saillenfait et al. 2011). Summarizing the above data it is explicit that phthalate esters caused dose-dependent fetal toxicity reflected in severe skeletal malformations and imbalance of bone homeostasis. These effects, observed in rodents such as other effects (e.g., developmental and testicular toxicity) observed in various animal species exposed to phthalates, can be considered relevant, also, for humans and are correlated with the human health (NTP-CERHR Expert Panel Report 2000; KEMI 2001). In fact, human exposures to environmental chemicals do not occur singly, but in aggregate complex mixtures. In addition, many phthalates display similar toxic effects as a group (Gray et al. 2000). The above considerations necessitate new approaches to assessing phthalate exposures and impacts to multiple phthalates in mixtures from multiple sources in order to define and prevent bone (and other target tissues) pathological conditions. The targets of phthalate esters on bone modeling and remodeling are schematized on Table 6.

Conclusions

The ability of certain chemicals to interfere with estrogen signaling is well documented. In addition to regulate the reproductive system/functions, estrogens are also important regulators of other cellular processes including bone metabolism.

There is compelling evidence regarding the capacity of EDCs to interfere with the bone remodeling and homeostasis through the modulation of the signaling pathways involved such as AhR, NRs, and ERs network cascades. The EDCs activating or antagonizing these receptors can lead to an imbalance hormone production (e.g., decreased steroid levels and increased estradiol ones) which could reflect on bone formation in pre- or postnatal stages or could be able to disrupt bone turnover in adult stages in mammalians. Regarding the effects on human health, it is well established that children and adolescents show a greater susceptibility to chemical toxicants (Goldman 1998). The available toxicity data demonstrated both in vitro and in vivo indicate that intensive exposure of

infants/children can approach toxic doses in rodents (Waring and Harris 2005). Nonetheless, this is a complicated area and there are not sufficient data so far on EDCs action on human bone tissue. On the other hand, it is comprehensible to assume that the adverse effects of EDCs, observed in human endocrine system, could provoke an impaired bone remodeling.

In this review, we have summarized the involvement of several chemicals with disrupting properties on bone homeostasis and metabolism. The examples of endocrine disruption cited above underscore the complexity of action of these chemicals in bone and point to a large number of potential molecular targets for xenobiotic disruption. Modern concepts regarding EDCs action evidenced that they can act as hormones through regulation of several metabolic routes such as histone deacetylase activity, mitogen-activated protein kinase activity, and DNA methylation status (Jones and Baylin 2002; Anway and Skinner 2006); these changes are crucial, because the receipt of a signal at an inappropriate developmental period may permanently influence gene expression by an epigenetic mechanism. In this context, it will be a note of interest to understand the intracellular pathways carried by EDCs in further field investigations. The understanding of the molecular and biochemical mechanisms of EDCs could provide a useful platform for the prevention of bone disorders and diseases.

Conflict of interest The authors declare that there are no conflicts of interest.

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