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Effects of diisononyl phthalate on osteopenia in intact mice

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ABSTRACT

Osteopenia is characterized by bone loss and deterioration of trabecular bone, which leads to osteoporotic fractures. This disease is highly prevalent in industrialized areas and is associated with exposure to endocrine disrupting chemicals (EDCs). Diisononyl phthalate (DINP) is one of these EDCs and is mainly used as a plasticizer in flexible polyvinyl chloride (PVC) products. Although it is well known that exposure to DINP is harmful to humans, no studies have been reported concerning its contribution to osteopenia. Therefore, in this study, we injected DINP (2, 20, and 200 mg/kg) into C3H/HeN mice for 6 weeks and found that the uterus weight, bone (femur and tibia) weight, and bone length of the DINP-exposed mice were reduced compared to those of the SHAM group. On the other hand, body weight, the serum alkaline phosphatase (ALP) and inorganic phosphorus (IP) levels in the DINP treated mice were increased compared with those of the SHAM group. The tartrate-resistant acid phosphatase (TRAP) activity (bone resorption marker) was increased and the bone alkaline phosphatase (BALP) activity was lowered by the treatment with DINP as compared with the SHAM group. Furthermore, the microarchitecture of the femur and tibia in the intact mice was destroyed by the DINP injection. The tissue volume (TV), bone volume (BV), BV/TV, bone surface (BS), BS/TV, trabecular thickness (Tb.Th), and trabecular number (Tb.N) were reduced and the trabecular pattern factor (Tb.Pf), structure model index (SMI), and trabecular separation (Tb.Sp) were increased by the DINP injection. The bone mineral density (BMD) of the femur and tibia was lower in the DINP group than in the SHAM group. These results indicate that DINP contributes to an increased risk of osteopenia via destruction of the microarchitecture and enhancement of osteoclast activity.

1. Introduction

The prevalence of osteopenia has increased all over the world and it has been recognized as a major health issue (Kanis, 1994). Osteopenia is a systemic skeletal disease characterized by a reduction of bone mass and microarchitectural deterioration of bone, with a consequent increase in bone fragility and susceptibility to fracture (Kanis et al., 2013). As one of the inflammatory diseases that occur in menopausal or post-menopausal women, osteopenia is associated with a reduction and depletion of the estrogen hormone (Mundy, 2007). The cessation of estrogen production in menopausal or post-menopausal woman induces an increase of bone turnover and bone resorption by osteoclasts, resulting in a decrease in bone strength and increase in bone fracture. The costs associated with osteoporotic fractures are becoming a major contributor to medical expenses worldwide (Park, 2008; Masi, 2008).

Since the Second World War, the rate of incidence of osteoporotic bone fractures in industrialized countries has been on the increase, though the reason for this is unknown. However, it has been suggested that exposure to endocrine disrupting chemicals (EDCs) or endocrine disrupting substances (EDSs) has adverse effects on bone and reproductive tissues, and that these effects are sex-dependent (UNEP/WHO, 2013). Concerns about exposure to EDCs are increasing, as they may have a direct impact on human health and wildlife (Lind et al., 1999). For example, in south-east Turkey during the period from 1955 to 1961, people displayed neurological, dermatological, and orthopedic abnormalities caused by the ingestion of hexachlorobenzene (HCB), a fungicide added to wheat seedlings (Cripps et al., 1984). As another example, Japanese people who ingested contaminated rice brain oil containing polychlorinated biphenyls (PCBs) exhibited symptoms such as intrauterine growth retardation, brown staining of the skin and mucous membranes, as in Addison's disease, natal teeth, widely open fontanelles and sagittal suture. Especially, this substance induced skeletal diseases, such as the unusual calcification of the skull (Miller, 1985). As demonstrated in these examples, EDCs are associated with various diseases, as well as bone disorders. If the exposure of humans to EDCs in developed countries were to increase, their contribution to

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bone disorders in humans would be likely to increase.

Phthalates, one type of EDC, are chemicals used as materials for personal care products, food packaging, children's toys, pharmaceuticals, nutritional supplements, cleaning materials, lubricants, insecticides, solvents, adhesives, paints, lacquers and building materials (Colborn et al., 1993; Chen et al., 2012). The esters of phthalates, such as phthalic acid, terephthalic acid, isophthalic acid, butylbenzyl phthalic acid, di-n-butyl phthalic acid, di-n-octyl phthalic acid, di-(2-ethylhexyl) phthalic acid, diethyl hexyl phthalic acid, dimethyl terephthalic acid, dimethyl isophthalic acid, and diisononyl phthalic acid (DINP), are used primarily as plasticizers in flexible polyvinyl chloride (PVC) products (Vamsee-Krishna and Phale, 2008). Especially, DINP exposure induces allergic airway inflammation in rat pups (Chen et al., 2015), asthma in normal mice (Hwang et al., 2017), and allergic dermatitis (Kang et al., 2016). Furthermore, the prenatal exposure of F1 female mice to a phthalate mixture made of 35% diethyl phthalate, 21% di (2-ethylhexyl) phthalate, 15% dibutyl phthalate, 15% diisononyl phthalate, 8% diisobutyl phthalate, and 5% benzylbutyl phthalate impaired their reproductive outcomes (Zhou et al., 2017a), and the exposure of F2 and F3 females to this mixture induced multigenerational and transgenerational effects on female reproduction (Zhou et al., 2017b).

Although the negative effects of DINP on women are known, to the best of our knowledge, no studies of the effect of DINP exposure on bone loss have been performed. We hypothesized that DINP might induce destruction of the microstructure of cancellous bone. Therefore, in order to evaluate the effect of DINP exposure on bone loss in intact mice, we exposed DINP (2, 20, and 200 mg/kg) to C3H/HeN mice.

2. Materials and methods

2.1. Reagents

Diisononyl phthalate (Fig. 1A) and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and Invitrogen Life Technologies (Carlsbad, CA).

2.2. Animals and experimental treatments

Eight-week-old female C3H/HeN mice (weighing 20–22 g) were purchased from Orientbio (Orientbio Inc., Iksan, Korea). The animals were housed in standard polycarbonate cages under controlled conditions (22 ± 2 °C, RH 50%–60%, and a 12-h light/dark cycle) and allowed free access to commercial rodent chow (DAE-HAN Biolink, Daejeon, Korea) and water. In the OVX animals, both ovaries were removed under Zoletil-induced anesthesia. The animals were allowed to recover from surgery for 5 days prior to the experiments. The mice were divided into 5 groups of 5 animals each as follows: a sham-operated control group, which were injected with PBS i.p.; a vehicle treated OVX group, which were injected with PBS, i.p.; and three DINP groups: a 2 DINP group, 20 DINP group, and 200 DINP group, in which the animals were administered 2, 20 or 200 mg of DINP/kg BW (body weight) daily (i.p.), respectively. The vehicle and DINP were administered for 6 weeks, and the body weights were recorded weekly. At the end of the 6-week treatment period, the animals were sacrificed by cervical dislocation. Sera were collected and stored at − 80 °C until use, and the uteruses, tibias and femurs were removed and weighed. The femur and tibia lengths were measured using a Vernier caliper. Also, the uterus was photographed with a digital camera.

All mice were treated in strict accordance with the Sunchon National University Institutional Animal Care and Use Committee's (SCNU IACUC) guidelines for the care and use of laboratory animals. All procedures were approved by the SCNU IACUC (permit number: SCNU IACUC-2017-01).

2.3. Measurements of serum IP, ALP, and LDH

The blood samples were maintained at room temperature for 1 h, and centrifuged at 5000 rpm for 5 min to obtain the serum. The serum was separated immediately and stored at − 80 °C. The serum calcium (Ca), inorganic phosphorus (IP), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and total cholesterol (TCHO) levels were measured using a diagnostic slide kit and an automatic analyzer (Fuji Dri-Chem, Fuji, Japan).

2.4. Measurements of TRAP, BALP and estradiol in serum by ELISA

The tartrate-resistant acid phosphatase (TRAP) activity (a marker of bone resorption) and serum estradiol (E2) levels were measured using a TRAP enzyme-linked immunoassay (ELISA) kit (USCN Life Science, Wuhan, China) and an estradiol ELISA kit (Calbiotech, San Diego, CA, USA), respectively. The bone alkaline phosphatase (BALP) levels were measured using a BALP ELISA kit (Elabscience, Wuhan, China).

2.5. Microcomputed tomography analysis

A morphometric analysis was conducted to determine the three-dimensional (3D) bone structure in vivo. We obtained the bone morphometric parameters of the distal femora cleaned of adherent soft tissues, including the tissue volume (TV), bone volume (BV), bone volume/tissue volume (BV/TV), bone surface (BS), bone surface/tissue volume (BS/TV), and trabecular thickness/separation/number (Tb.Th, Tb.Sp, and Tb.N), after scanning with a SkyScan 1272 apparatus (SkyScan, Kontich, Belgium) and analyzing the volume of interest. The regions of interest for analysis were the proximal tibia and distal femur metaphysis. For the quantification of the trabecular volumetric mineral

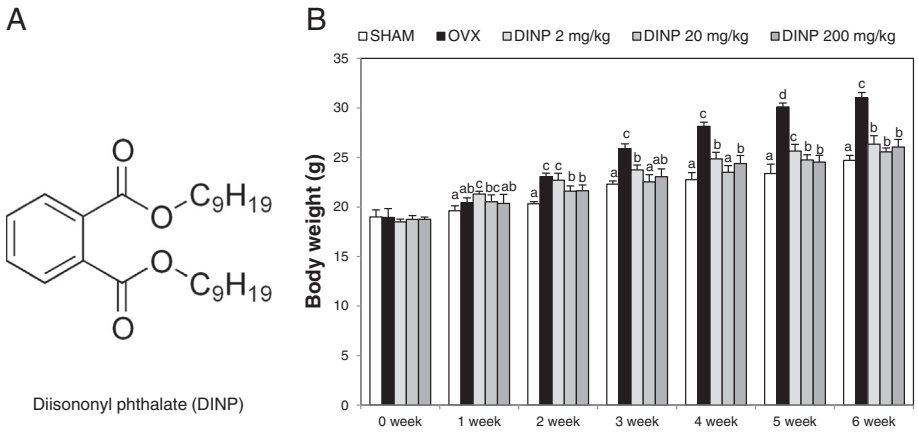

Fig. 1. Effect of DINP on body weight. (A) Molecular structure of DINP (diisononyl phthalate). C3H/HeN mice were treated with DINP for 6 weeks. (B) Body weight was measured at 24 h after the last treatment. [a, b, c, d] The means not sharing a common letter are significantly different among groups at $p < 0$ 05 by Ducan's multiple-range test.