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Research paper

Sub-NOAEL amounts of vinclozolin and xenoestrogens target rat chondrogenesis *in vivo*

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ABSTRACT

Several endocrine disrupting compounds (EDC) elicit skeletal dysgenesis at pharmacological doses. We have investigated the impact of doses below the "No Observed Adverse Effect" (NOAEL) for vinclozolin (V), an anti-androgenic fungicide, alone or associated with xenoestrogens (Genistein, G and bisphenol-A, BPA). V, G, BPA and their combinations were administered orally to female Wistar rats during gestation and lactation. F1 and F2 offspring were investigated for skeletal anomalies at post-natal days 30, 110 (d30, d110). Skeletal development was monitored by measuring caudal vertebrae and long bones dimensions by X-ray micro-CT-scan. A significant increase in Inter Transverse Apophysis (ITA) distance at the upper head of caudal vertebrae, associated with a reduction in vertebral body height was observed in treated F1 females, but not males. Histometrical analysis of vertebral body growth plate cartilage was performed on serial sections of caudal vertebrae. F1 females but not males showed a diminution in growth plate thickness, with greater impact on the hypertrophic zone. All effects were maximal at d30. Effects on ITA width persisted until d110 while effects on growth plate disappeared. These effects were essentially vinclozolin or BPA-dependent. F2 animals were not affected. Our data suggest that vinclozolin and xenoestrogens act as cartilage developmental disruptors. We suggest that present NOAEL values for these compounds, and EDC at large, might be reconsidered using gestational exposure models. Finally, micro CT-scan appears a valuable non-invasive technique to detect EDC effects on live fauna.

1. Introduction

Endochondral bone formation and growth have been shown to be under the control of endocrine factors and hormones including IGFs, growth hormone, thyroid hormones, vitamin D metabolites and sex steroid hormones [1–3]. Estrogens induce growth plate (GP) senescence, causing exhaustion of the chondrocytes pool and epiphyseal fusion [4]. Very few studies have been performed on the effects of environmental toxicants acting on cartilage as endocrine disrupting compounds (EDC), such as xenoestrogens and pesticides. Vinclozolin (Ronilan®) is a dicarboximide fongicide widely used in agriculture since the 90's. It has been banned in Europe since 2006, but not in other countries and illegal trade and use remain a possibility. The "no observed adverse effect level" (NOAEL) is 12 mg/kg/day in Wistar rats [5]. A lower figure (1.2 mg/kg/day) was later proposed by the US EPA without specifying the rat species used in the analysis [6]. Vinclozolin is weakly estrogenic "per se" while its metabolite M2 is an anti-androgen closely resembling Flutamide®. Genistein is an isoflavonoid from Leguminosa, essentially present in soy-derived products. According to the AFSSA report, daily intake is below 1 mg/kg/day except for infants fed soymilk (up to 5 mg/kg/day) or adult soy-derived products consumers (up to 60–100 mg/kg/day) [7]. NOAEL for genistein is 100 mg/kg/day in Wistar rats [8]. Bisphenol-A (BPA) is an alkylphenol used in food packaging (metal can coating, plastic containers) and dentistry cements. BPA transfers easily into foodstuffs and infants are exposed through the use of polycarbonate bottles.

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BPA NOAEL is still a matter of controversy. The classic NOAEL values of 5 mg/kg/day in Sprague Dawley rats [9] and "inferior to 1.5 mg/kg/day" in a gestational model of Wistar rats [10] have been extensively challenged. Recent works suggest a value closer to 2–20 μg/kg/day [11]. However most of the recent studies do not specifically state a NOAEL nor do they present dose–response curves for most of them (reviewed in Ref. [12]). Hence we can only say that BPA NOAEL should be in the range of 5 μg to 5 mg/kg/day. Both Genistein and BPA are xenoestrogens with affinities for the estrogen receptors (ERα, β). Estradiol, Genistein and BPA affinity for ERα in rats are 0.1–0.2 nM [13], 10–20 nM [14] and 0.23–0.33 μM [15], respectively.

The literature reports vertebral dysmorphism in fish treated by ethynyl-estradiol but not BPA [16]. A variety of pesticides elicit cartilage anomalies in mammals at pharmacological doses (20–300 mg/kg/day) [17,18]. In addition, a vertebral anomaly, in the form of visible "annealed tails", was observed in Wistar rats exposed to high doses of vinclozolin (V, 30 mg/kg/day) using a model of *in-utero* and lactational exposure (Fig. 1) [19]. Noteworthy, the lower exposure used in these studies (1 mg/kg/day) elicited non-visible but palpable annealing of the tails of treated animals. Taken together, these data suggest that EDC might disrupt skeletal development. Work on digit development using ER agonists and antagonists also support our hypothesis of such molecules being "cartilage disruptors" [20], as does a recent study based on *in utero* exposure to environmental endocrine active substances [21].

The latter results, added to other observations on rat fertility disruption, led to the constitution of a multicentric joint project to study the developmental toxicology of vinclozolin alone or combined with xenoestrogens in the rodent gestational/lactational model described by Eustache et al., [19]. Our laboratory focussed on the effects of very low doses and cocktail effects on rat cartilage development in vertebral bodies and long bones. The first study (National Research Program on Endocrine Disruptors, PNRPE-2006, Auger, J. coordinator) used doses under NOAEL values for these compounds (sub-NOAEL, see methods). The second study (Continuous Impact to Mixes of Endocrine disruptors, CIME-2009, Canivenc-Lavier, M.C. coordinator) used combinations of doses far below NOAEL doses (far-NOAEL, see methods) [22]. Briefly, we used doses 100-fold below NOAEL for genistein, 12-fold and 1200-fold below NOAEL for vinclozolin while the ratio below NOAEL for BPA awaits a definitive NOAEL value. If we accept the current 5 mg/kg/day, the dose ratio is 1000-fold below NOAEL [9].

Our low-dose studies did not reproduce the naked eye-observable "annealed tails" elicited by vinclozolin at 30 mg/kg/day, but this "annealing" was still clearly detectable by palpation in F1 animals exposed to vinclozolin alone (1 mg or 10 μg/kg/day) or combined with xenoestrogens in accordance with the above cited previous data [19]. These tail deformities were associated with radiological and histological modifications of caudal vertebral bodies and their GP cartilage.

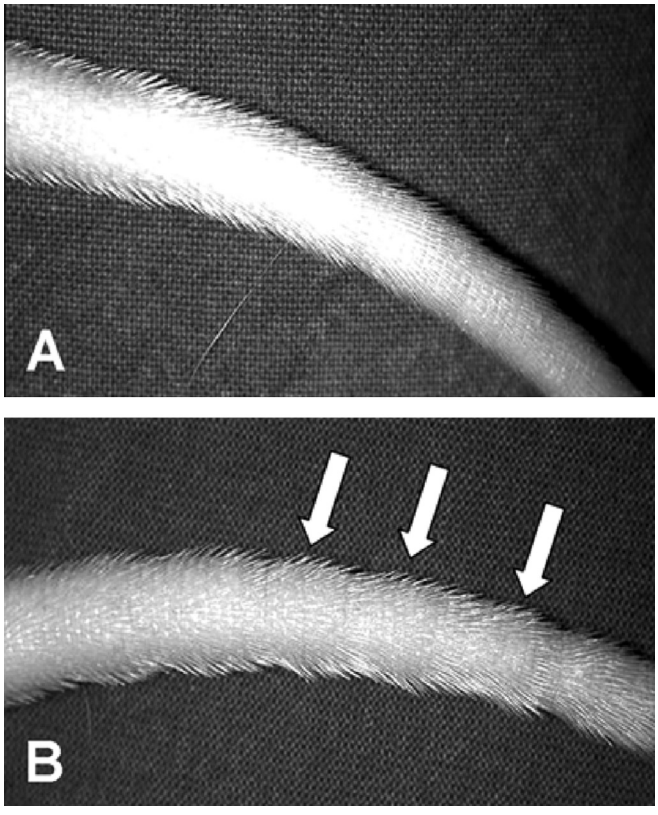

Fig. 1. Wistar female rats were treated or not with vinclozolin (30 mg/kg/day) during gestation and breast feeding. Representative pictures of offspring tails are shown: (A) unexposed offspring male. (B) exposed offspring male. (communicated by J. Auger et M.C. Canivenc–Lavier, 2002, and Ref. [19]).

2. Materials and methods

2.1. Chemicals

Chemicals, including vinclozolin (V, purity 99.6%; Ronilan®), bisphenol-A (BPA, purity 99%) and genistein (G, purity 99%) were from Sigma France.

2.2. Animals and exposure protocols

2.2.1. Animals

The local authorities approved all procedures involving Wistar Han rats, according to the French Ministry of Agriculture ethical guidelines for care and use of laboratory animals and Good Laboratory Practices. Investigation agreements were given by the Universite de Bourgogne Ethics committee and were registered under: PNRPE: Jan/23/2006 *n*° E0607 and CIME: Dec/16/2009 *n*°A1909.

A total of 60 specific-pathogen-free (SPF) female and 60 male Wistar Han rats at 8 weeks of age (Harlan France Sarl; Gannat, France) were acclimatized to SPF housing conditions (22 °C room temperature with 55% relative humidity and a 12-h light/dark period) for 4 weeks before mating. Cages and bottles were made of polypropylene to avoid any contamination by bisphenol A or phthalates and water was filtered through charcoal to eliminate any pesticide or active endocrine contaminant. Animals were fed a purified phytoestrogen-free diet (INRA, France) [23] and allowed ad libitum access to charcoal-filtered water as previously described [19]. All drugs were given by oral gavage of gestant and lactating mothers. Pup weaning was at d21, then pups were sacrificed for analysis at d30 and d110 after birth.

2.2.2. Sub-NOAEL protocol

Females were examined daily after mating to determine the first gestational day (d0 or G0) by examining the presence of spermatozoa in vaginal smears or the vaginal plug. Dams were randomly allocated to four groups corresponding to control (C), genistein 1 mg/kg/day (G), vinclozolin 1 mg/kg/day (V1), and genistein/vinclozolin (GV1) groups (15 rats/group). This corresponded to 100-fold below NOAEL for G and 12-fold below NOAEL for V. At parturition, the litters were weighed, sexed and standardized at ten pups. From birth to weaning (d21 or G21), dams daily received xenohormones at 1 mg/kg/day oral dose, alone or in combination.