ORIGINAL RESEARCH ARTICLES

IMPACT OF THREE ENDOCRINE DISRUPTORS, BISPHENOL A, GENISTEIN AND VINCLOZOLIN ON FEMALE RAT ENAMEL

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Key words
Endocrine disruptor, bisphenol A, genistein, vinclozolin, enamel, hypomineralization, female rat.

Abstract
Concerns about the potential adverse effects of endocrine disruptors (EDs) have been increasing over the last three decades. Bisphenol A (BPA), genistein (G) and vinclozolin (V) are three widely used EDs sharing similar effects. Since populations are exposed to many diverse EDs simultaneously, we demonstrated recently their impact alone or combined on male rat tooth enamel. The purpose of this study was therefore to assess their effects on female rat tooth enamel in order to understand why they are differentially sensitive. Rats were exposed daily in utero and after birth to low doses of EDs during the critical fetal and suckling periods when amelogenesis takes place. Enamel of rats exposed to EDs presented opaque areas of hypomineralization. The proportion of affected rats was the highest in the groups of rats treated with BPA alone and higher in males than in females (in all the groups). Comparison of enamel key gene expression levels showed modulations of *Klk4* and *Enamelin* in males but no significant variations in females. These findings show that female rats are less affected than males by the three EDs chosen in this study and suggest that enamel hypomineralization may differ between males and females.

Introduction
An endocrine disruptor (ED) is currently described at the international level as an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) population (1). Until June, 2015, 1000 molecules have been identified as EDs on the TEDX (The Endocrine Disruptors Exchange) list. Due to the multiplicity of areas of their use, EDs are found ubiquitously throughout our daily environment. Populations are consequently exposed to many diverse EDs simultaneously. It is therefore important to explore the effects of combinations of EDs with similar described effects.
Bisphenol A (BPA), used in the manufacture of epoxy resins and polycarbonate plastics, is an exemplary widespread ED. Many experimental studies have shown that BPA affects diverse organs. It has been described for its estrogen-mimetic and anti-androgenic effects (2). Genistein (G), a phytoestrogen found in legumes, presents structural and hormonal properties similar to estrogens and has also anti-androgenic effects (3). Vinclozolin (V) is

a fungicide with anti-androgenic effects; it can bind both androgen receptor and estrogen receptors with lower affinity (4).
Concern arises recently on their adverse effect on tooth. BPA disrupts amelogenesis by targeting the enamel matrix protein (EMP) coding gene *Enamelin* and the protease *Kallikrein 4 (Klk4)*, leading to enamel hypomineralization (5). G, V and the combination of the three EDs do not have the same effects as BPA (6). The results of all these studies have been described in male rats. Since EDs disturb mainly the hormonal system, the following question emerges as a consequence: Are female teeth impacted in the same manner as those of males after exposure, with the same conditions, to the same EDs? And if not, what is the underlying explanation?

Material and methods

Animals and biological samples

The animals used in this study were bred as previously described (5). Briefly, Wistar Han rats were purchased from Harlan France Sarl (Gannat, France). All animals were maintained in accordance with the French Ministry of Agriculture guidelines for care and use of laboratory animals (authorization number B2 231010EA). Cages and bottles made of polypropylene were used to avoid any contamination by BPA or phthalates, and drinking water was filtered through charcoal to eliminate any trace of phytoestrogens and pesticides. On gestational day 1 (E1), the dams were randomly divided into six groups. From gestational day 1 until weaning (P21), pregnant females were orally administered one of the following treatments daily: G/V, BPA, G/BPA, V/BPA, G/V/BPA at doses of 1 mg/kg/day G (Sigma-Aldrich), 10 µg/kg/day V (Toxalim laboratory, Toulouse, France) and 5 µg/kg/day BPA (Sigma-Aldrich) in 0.4 mL/kg body weight of corn oil; the control group was administered corn oil alone. After weaning, young female rats were similarly exposed to same mixtures daily until sacrifice at P30.

RNA extraction and real time-qPCR (RT-qPCR) analysis

Total RNAs were extracted using Tri-Reagent (Euromedex, Strasbourg, France) according to the manufacturer's instructions. Reverse transcription was carried out with 1 mg of total RNA for 45 min at 42 °C, using the oligo-

Gene	Amplicon size	Primer sequences
Rs15	315 pb	5'-GGCTTGTAGGTGATGGAGAA-3' 5'-CTTCCGCAAGTTCACCTACC-3'
Gapdh	260 pb	5'-GACCCCTTCATTGACCTCAACTAC-3' 5'-AAGTTGTCATGGATGACCTTGGCC-3'
Amelogenin	271 pb	5'-ACACCCTTCAGCCTCATCAC-3' 5'-GAGAACAGTGGAGGCAGAGG-3'
Enamelin	439 pb	5'-CATGTGGCCTCCGCCAGTCC-3' 5'-GTCATCTGGGGGCGGGTCCT-3'
Ameloblastin	258 pb	5'-TGCAGCCTCACCAGCCAGGA-3' 5'-CCCGAGACAGCGAATGGGCG-3'
Tuftelin	202 pb	5'-CTCCCCTGTCCGCAGCAAGC-3' 5'-GGCGTCCATGTGCTGCTGGT-3'
Amelotin	379 pb	5'-GCAACAAAACCGACTCCAG-3' 5'-CTCCATTCTGCACATCTGG-3'
Mmp20	320 pb	5'-CTGGGCCTGGGCCATTCCAC-3' 5'-CTGGTGATGGTGCTGGGCCG-3'
Klk4	320 pb	5'-GCATCCGCAGTGGGTGCTGT-3' 5'-CACACTGCAGGAGGCTGGGC-3'

Table 1: Primer sequences used for RT-qPCR analyses.

dT mix of random primers according to the manufacturer's instructions (Superscript II® - Invitrogen). An Opticon Monitor device (Bio-Rad Laboratories, Hercules, CA) was used for RT-qPCR. Details of the primers and the corresponding amplicon sizes are presented in Table 1.

Statistical analysis

The results of all experiments are reported as means ± SD. Data being not normally distributed, the two-tailed non-parametric Mann-Whitney test was used for comparisons (of each test group with the control). GraphPad Prism software version 4.0 (La Jolla, CA) was used for data analysis and values were considered significantly different (*) when p≤0.05.

Results

The highest prevalence of affected female rats was only 31%

Incisors of female rats exposed to three EDs from E1 to P30 were carefully observed and scored as previously described (5). Control rats presented yellow-brown incisors, whereas 20% to 31% of rats exposed to EDs exhibited white opacities on their mandibular incisors. The prevalence of affected rats differed according to the treatment (Table 2): the highest prevalence, 31%, was in the group exposed to BPA alone. The teeth in this group displayed an anarchic distribution of white spots (7). In the other groups, about 20% of the rats were affected (Table 2).

Control (C)	0%
Genistein Vinclozolin (G/V)	20%
Bisphenol A (BPA)	31%
Genistein Bisphenol A (G/BPA)	20%
Vinclozolin Bisphenol A (V/BPA)	20%
Genistein Vinclozolin Bisphenol A (G/V/BPA)	20%

Table 2: The prevalence of affected female rats, after chronic low dose exposure to genistein, vinclozolin, bisphenol A and their combination, was systematically compared to the control one (n=8). Control rats were never affected.

Chronic low dose exposure to genistein, vinclozolin and bisphenol A do not affect EMP-nor protease- coding genes in female rats

The levels of the mRNAs encoding the major EMPs, Amelogenin, Ameloblastin, Amelotin, Enamelin, and proteases, Mmp20 and Klk4 did not varied between rats exposed to the various combinations of G, V and BPA. Of notice, RNA levels presented important variations within the same group making the difference with the control group non significant (Figure 1).

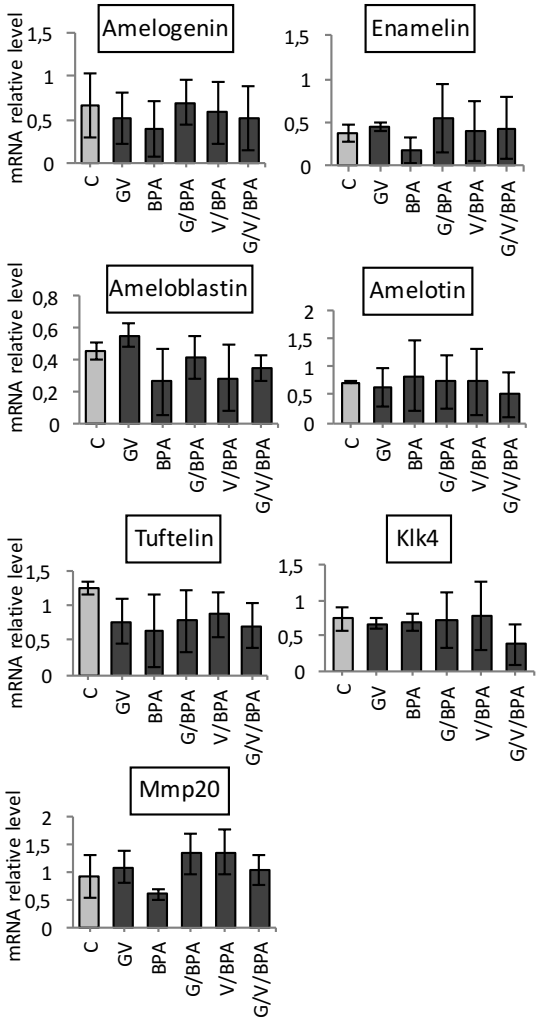

Figure 1: Expression of enamel genes analyzed by RT-qPCR. Analysis of mRNAs extracted from microdissected dental epithelium of female rats didn't show any significant difference of enamel genes expression in the various treated groups compared to the control one. All data are means ± SD for experiments performed in triplicate.

Discussion

Concerns about potential adverse effects of exposure to EDs are growing day after day. We recently demonstrated that continuous exposure to low-dose BPA affects enamel mineralization in 75% of male rats (5). The window of sensitivity to BPA covers the perinatal period, and therefore BPA is able to act as an enamel hypomineralizing factor. As BPA-exposed rat enamel and MIH affected teeth share similar structural and biochemical properties, BPA can be considered to be

a causative agent of MIH (molar incisor hypomineralization) (5). This recently described pathology is characterized by irreversible white opaque spots on the enamel of the first permanent molars and incisors. It affects roughly 18% of children between 6 and 9 years old (8). In this study, we observed that BPA disturbed enamel mineralization in only 31% of female rats whereas it was the case of 75% of males (5,6,7). Regarding females, they were less affected than males not only in BPA group but in all combination groups (GV, G/BPA, V/BPA, G/V/BPA). In addition, molecular analysis results in females showed significant inter-individual variations making differences non statistically significant. The estrous cycles and the consequent hormonal changes in females could interpret these observations. However, the general response trend in females is to the decrease of total enamel proteins and proteases studied. This difference let us suggest that males are more susceptible than females to endocrine disruption during fetal life and early postnatal weeks.

As already known, the fetal period may considerably influence the development of pathologies during adulthood. In case of ED exposure during this period, several studies report variable susceptibility depending on sex raising the question of steroid receptor involvement. Overall, males subject to non-optimal conditions in utero tend to be more susceptible to various diseases, including neurobehavioral disorders (9). It seems that the placenta has a greater capacity to counteract environmental fluctuations for female than male fetuses (10,11). Moreover, it was also demonstrated that perinatal exposure to BPA could jeopardize the sexually dimorphic ability of the liver to metabolize drugs and steroids (12). It has been described that treatment with BPA decreases the rate of BPA glucuronidation enzymes (UGT2B1) in liver microsomes of male rats but not females (13).

By comparing our results to the MIH literature data concerning the prevalence differences between boys and girls, we notice that it would be necessary to conduct more studies that will consider this point to be able to make a conclusion on the whole population. The data published until now do not show clearly preferential impacts in boys although both sexes do not have the same impact profiles (14). These results raise the question on the BPA signaling pathway and more generally on the steroid, androgen and estrogen pathways, potentially different between males and females. Although steroid concentrations, estrogen and testosterone, reach their maximal level during the puberty, their circulating levels during the fetal and the perinatal are also important (with a decrease during the childhood). Thus steroids are involved in the development of many organs that EDs may disrupt.

BPA mechanism of action is not entirely exerted through ERα in ameloblastic cells especially on gene expression modulations, indicating that there are other additional pathways affected by BPA (7). Initially, an ED was regarded as a chemical compound able to bind to nuclear hormone receptors, in particular ERs, and thereby act as an agonist or antagonist of the endocrine system. In that sense, EDs (including BPA) have been shown to bind to many receptors explaining their broad effects (15). In addition, following more research in the field, it became evident that these xenobiotics could affect the endocrine system at several points along endocrine pathways, for example steroid biosynthesis and metabolism (1). These impact points may be also different between males and females.

The combination of BPA with two other EDs, genistein and vinclozolin, sharing similar effects has shown less impact on enamel mineralization than BPA alone (6). It is actually known that EDs do not necessarily share structural properties and even if they do, they are able to act through different signaling pathways and receptors that have not been exhaustively characterized. Consequently, the effects of combinations of EDs are unpredictable as they results from a combinatory of receptor activations. EDs can thus potentiate each other's actions or, on the contrary, suppress them (1).

Conclusions

To conclude, we report that BPA impacts enamel mineralization. Percentage of female rat affected by BPA is less than the one of male rats. We also demonstrate that combination of BPA with two other EDs, genistein and vinclozolin, has less activity than BPA alone and that the combination has also less effect on females than on males. These differences between males and females raise again the issue of sexual dimorphism.

Finally, the study of the impact of new EDs and their combination on enamel formation is extremely interesting even essential to define precautions avoiding adverse effects. Re-

garding the impact on the tooth, the study of substance combinations is not limited to EDs but could be extended to all substances with hypomineralization effect even all substances that affect the mineral.

Acknowledgements
We acknowledge the French Institute of odontology research (IFRO) for funding this work. We thank too Raymond Bergès, Laurence Decoq, Bruno Pasquis, Elise Lalarme (UMR 1324 INRA, Dijon) for animal breeding.

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