Different exposure windows to low doses of genistein and/or vinclozolin result in contrasted disorders of testis function and gene expression of exposed rats and their unexposed progeny

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Original Article

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Abstract

Living species including humans are continuously exposed to low levels of a myriad of endocrine active compounds that may affect their reproductive function. In contrast, experimental designs scrutinizing this question mostly consider the gestational/lactational period, select high unrealistic doses and, have rarely investigated the possible reproductive consequences in the progeny. The present study aimed at assessing comparatively a set of male reproductive endpoints according to exposure windows, gestational/lactational versus pre-pubertal to adulthood, using low doses of endocrine active substances in male rats as well as their unexposed male progeny. Animals were orally exposed to 1mg/kg bw/d of genistein and/or vinclozolin, from conception to weaning or from prepuberty to young adulthood. A number of reproductive endpoints were assessed as well as testicular mRNA expression profiles, in the exposed rats and their unexposed progeny. Overall, the low dosage used only affected weakly most of classical reproductive endpoints. However, the gestational/lactational exposure to vinclozolin alone or combined to genistein significantly delayed the puberty onset. Contrasting with the gestational/lactational exposure, a decreased sperm production was found in the animals exposed to genistein and vinclozolin from the pre-pubertal period but also in their progeny for vinclozolin and the mixture. The expression level of several genes involved in meiosis, apoptosis and steroidogenesis was also affected differentially as a function of the exposure window in both exposed rats and unexposed offspring. We also provide further evidence that doses of endocrine active substances relevant with human exposure may affect the male reproductive phenotype and testicular transcriptome in the exposed generation as well as in the indirectly exposed offspring.

***Key words (5)*:** endocrine disruption, exposure window, male reproduction, mRNA, multigenerational

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Animal care

All applicable international, national, and institutional guidelines for the care and use of the animals were followed. All animals were treated humanely and suffering was minimized. All procedures involving rats were approved by the Ethics Committee of Burgundy University.

Introduction

Low doses, mixtures and long-lasting periods characterize environmental exposure to endocrine disrupters (EDs) in humans. By contrast, the vast majority of experimental schemes were most often based on high unrealistic doses of EDs, generally for a single compound studied with an in utero/perinatal exposure window. One consequence of these studies is that the gestational and lactational periods are more generally considered the prominent developmental periods where EDs induce both contemporary and future physiological dysfunctions, noticeably of the male reproductive system (Atanassova et al., 2000; Christiansen et al., 2010; Peretz et al., 2014), this assertion being also one of the main conclusions of human studies (Sweeney et al., 2015). However, continuous EDs exposures encompass the pubertal period where the increase in the production of gonadal steroids underlying change of male sexual behaviour and sperm production may be disrupted (Evuarherhe et al., 2009; Woodman, 1997). In addition, possible modulations of the effects due to the continuity of exposure have rarely been considered. In a previous study, using a life-long exposure to the phytoestrogen genistein, a dietary compound present in several legumes, especially soy, and, the antiandrogenic fungicide and food contaminant vinclozolin, alone and in association, we reported various disorders of the male reproductive development and testis function (Eustache et al., 2009). In the same study, we also showed that mRNA expression profiles in the adult testis were significantly and differently modified according to the exposure modalities with functional clustering of the genes affected into ontological families.

Besides, a growing number of studies have suggested that EDs could promote the epigenetic transgenerational/multigenerational inheritance of disease and phenotypic variation including male reproductive disorders (Doyle et al., 2013; Skinner et al., 2013). However, most of these studies rely on high level ED exposure with an exposure window limited to the gestational period. Thus, studies with more realistic exposure modalities are required (Brehm and Flaws 2019).

The aim of the present investigation was to assess comparatively the effects of a gestational/lactational (GL) versus pubertal and beyond exposure (puberty to adulthood, PA) to low dose genistein and/or vinclozolin using various endpoints related to the male genital tract as well as testicular mRNA expression in the exposed generation as well as their unexposed progeny. We selected the dose of 1 mg/kg bw/d for both compounds according to the reproductive effects reported previously for this dosage (Eustache et al., 2009). This dosage corresponds to a level of genistein similar to that present in soya-based diets (Tanaka et al., 2008). For vinclozolin, it is similar to the US Environmental Protection Agency NOAEL, no observed adverse effect level (1.2 mg/kg bw/d, based on a combination of chronic toxicity, carcinogenicity and toxicity reproduction in rats; US EPA, 2003) while above the level of human food contamination with vinclozolin residues. The estimated acceptable daily intake according to the European Food Safety Authority, EFSA is 0.005 mg/kg bw/d (European Food Safety Authority, 2008).

Materials and Methods

Chemicals and doses used

Both compounds, genistein (greater than or equal to 99% purity) and vinclozolin (greater than or equal to 95% purity) were obtained as previously reported (Eustache et al., 2009). The selected doses were based on the results of Eustache and colleagues (2009) providing evidence that a mixture of genistein and vinclozolin (both at 1 mg/kg bw/d) chronically administered by gavage had a more severe impact on a number of male reproductive endpoints than a markedly higher dose of each compound separately. Therefore, in the present study investigating comparatively the role of exposure windows on male reproductive function as well as the possibly different reproductive consequences in the subsequent unexposed male offspring, we selected the single dose of 1 mg/kg bw/d for both genistein and vinclozolin, administered alone or in association.

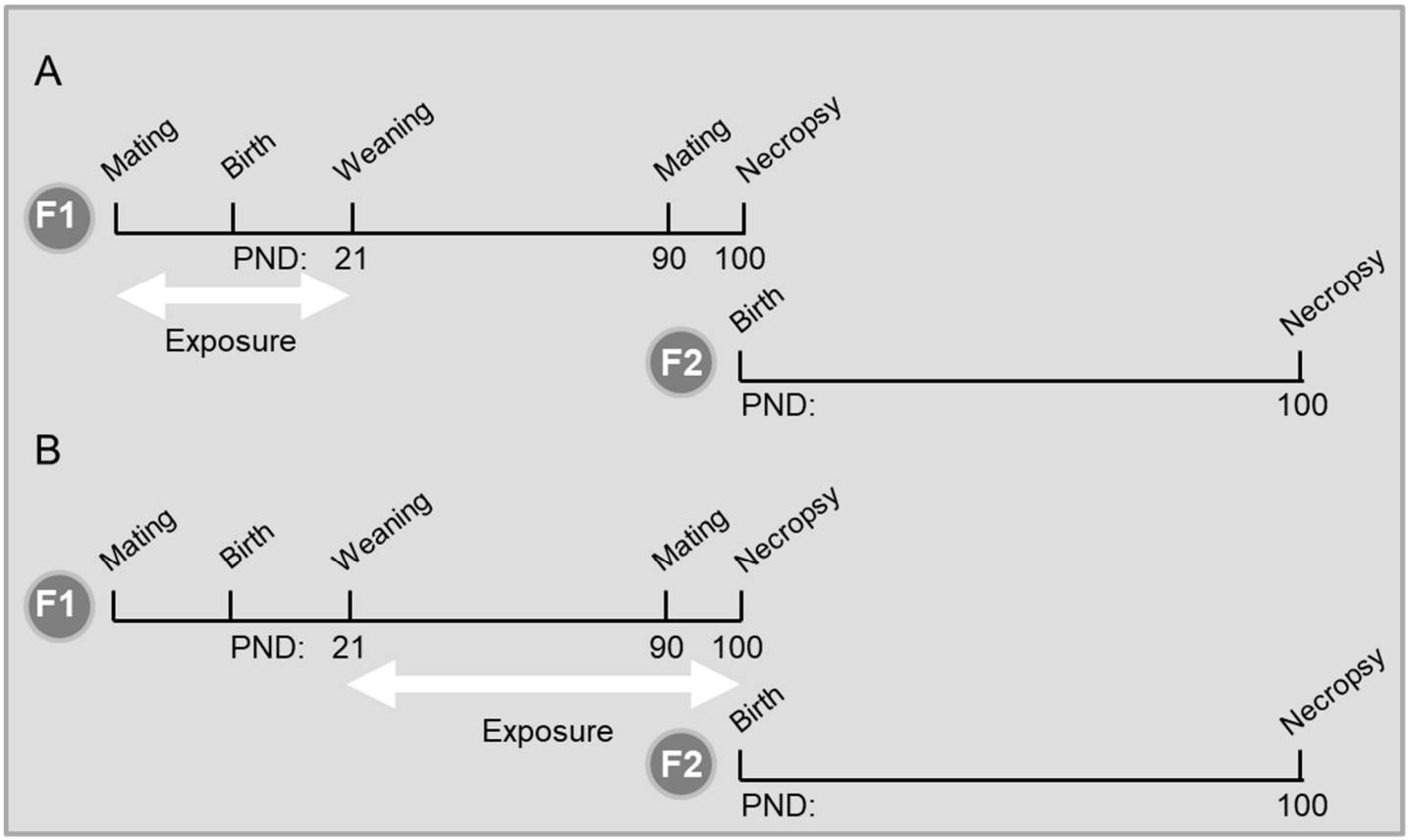
Animals and regimens

All animals were treated humanely and suffering was minimized. All procedures involving rats were approved by the Ethics Committee of Burgundy University and conducted in accordance with French guidelines for the care and use of laboratory animals. The selected animals, the acclimatization period, animal facility and feeding conditions were the same as in Eustache et al (2009). Briefly, specific pathogen-free female and male 8 weeks of age Wistar rats, obtained from Harlan France Sarl (Gannat, France), were maintained under controlled conditions at 22°C ambient temperature with 55% relative humidity and a 12h light and 12h dark cycle. From the acclimatization period, they were fed a soy-free purified diet (18% casein, 46% starch, 23% sucrose, 5% corn oil, 2% cellulose, 5% mineral mixture, and 1% vitamin mixture; Harlan Teklad, Gannat, France) and supplied with water *ad libitum*. Cages and bottles were made of polypropylene to avoid any contamination by bisphenol A or phthalates and the water was filtered through charcoal to eliminate any pesticide or active endocrine contaminant. Aspen sawdust was used as bedding, to prevent exposure to endocrine-like chemical residues.

Animal husbandry and exposure conditions

Females were mated with male rats as previously described (Eustache et al., 2009). They were examined daily and caged separately when a vaginal sperm plug was observed (considered as the day of conception). On the day of parturition (PND, postnatal day 0), pups were weighed and identified by ear and paw tags. All litter sizes were standardized to 12 offspring. Chemicals were dissolved in corn oil (Carrefour, Dijon, France) and administered by gavage (2 mL/kg bw). Control animals were treated with the vehicle alone. Two exposure window modalities were selected (Fig. 1): i) a gestational/lactational exposure window (GL) where dams were exposed daily to genistein or vinclozolin or their association, from conception (gestational day 1, GD1) to weaning (PND21) or, ii) a pre-pubertal to adulthood exposure window (PA) where male rats were exposed daily to genistein or vinclozolin or their association immediately after weaning until PND100.

Fig.1 Experimental design. A. Gestational-lactational exposure, from GD1 to PND21 (GL). B. Pre-pubertal to adulthood exposure, from PND21 to PND100 (PA). The F1 exposed male rats were mated with naive females to obtain an F2 that was maintained until adulthood without exposure. Necropsies were performed at PND100 in both generations. The 3 exposure modalities consisted in: i) genistein alone (G, 1 mg/kg bw/d) or ii) vinclozolin alone (V, 1 mg/kg bw/d) or, iii) a 1:1 genistein/vinclozolin mixture (GV, 1 mg/kg bw/d genistein + 1 mg/kg bw/d vinclozolin).



Anogenital distance, reproductive abnormalities rate and puberty timing

The anogenital distance (AGD) was measured with a digital caliper at weaning on all male rats as previously described (Auger et al., 2013), and at PND21 on 50 rats per exposure group, by the same observer blind of exposure groups. Relative AGD (AGD/bw) was calculated. All macroscopic developmental anomalies of the reproductive tract (undescended testis, hypospadias…) and anomalies observed after dissection in the adult (i.e., epididymal cysts) were scored in order to determine their overall rate. The time of puberty onset was defined as the post-natal day of complete preputial separation (Korenbrot et al., 1977). It was assessed on a random subset of 20 animals per exposure group. For all these assays, the observers were blinded to exposure condition to avoid bias.

Mating and fertility

Fertility and reproductive performance were assessed only for F1 male rats. On PND90, 10 males per group were selected for mating with unexposed acclimatized females (Harlan France Sarl, Gannat, France): each male was caged with one female for

four days. As described above, females were inspected daily and caged separately until parturition when a vaginal sperm plug was found. The fertility endpoints used were the mating index (number of mated females / number of females cohabited with males x100), the fertility index (number of pregnant females / number of mated females x100), the litter size and mean weight per neonate, the sex-ratio at birth, and the percentage of post implantation loss (number of embryonic scars / number of embryonic buttons and scars x100).

Sacrifice, tissue collection, weighing and epididymal (cauda) sperm number

At PND 100 and for each exposure modality, 20 exposed males were anesthetized using isoflurane (2.5%) and killed by thoracic cage opening. All male pups stemmed from exposed fathers were raised until PND100 and sacrificed in the same manner. For each animal (F1 or F2), both testes, epididymis and seminal vesicles, ventral prostate and liver were excised and trimmed of fat, then weighed (Precisa, model 125A; Poissy, France). Relative weights were calculated. Epididymal sperm number was assessed using computer aided sperm analysis (CASA) technology as previously described (Eustache et al., 2009) in a random sample of 10 animals per exposure group (F1 and F2).

RNA preparation

RNA was prepared from individual testes obtained from six animals in each group, using classical TriZol extraction procedures; briefly, testes were directly put in 1 mL TriZol-containing tubes, the tissue was minced with a sterile steel bead and 2 Hz shaking during 1 minute. Then 200 µL of chloroform was added, the tube was vortexed and centrifuged at 5000 g during 5 minutes. The supernatant (600 µL) was precipitated with 500 µL of cold isopropanol; after centrifugation, the pellet was rinsed with 70% Ethanol, dried 10 minutes under a fume hood and resuspended in ~100 µL of RNAse-free water. RNA was quantified and its quality was evaluated using a Bioanalyzer (Agilent). All RNAs had a RIN (RNA Integrity Number) > 8. Pools of RNAs from six testes were prepared with strict equilibration of their quantity to ensure equal representation of each individual RNA.

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

Reactions were carried out in a 10 µL volume using a Roche Lightcycler (Roche, Meylan, France) and an Invitrogen Sybergreen quantitative PCR kit (Invitrogen, Cergy-Pontoise, France). After 5 minutes at 95°C, each reaction was carried out for 45 cycles (95°C for 10 seconds, 55°C for 15 seconds, and 72°C for 15 seconds when fluorescence was measured). This was followed by progressive melting by increasing the temperature from 65°C to 97°C during 10 minutes, with continuous fluorescence capture. The resulting melting curve was considered satisfactory if one single peak was visible on its derivative. In addition, PCR products were subjected to electrophoresis on an agarose gel to check for a single product of the expected size. *Sdha* was used as an endogenous control and for normalization of gene targets. For both generations, six males from each group were evaluated in triplicate. The comparative Ct method was used to calculate relative expression reported as mean relative mRNA expression in arbitrary units. The testicular transcriptome analysis was carried out for a set of 11 genes crucially involved in the meiosis, apoptosis and steroidogenesis steps (see Table 1 reporting the complete primer sequences for the selected genes).

Table1. Primer sets used for quantitative real-time PCR

Gene name	Forward primer (5'-3')	Reverse primer (3'-5')	Product length(bp)	Tm (ºC)
Meiosis				
Daz	TCTTCATCAGCAACCACCAG	GACAAATCCATAGCCCTTCG	195	60
Stra8	CTCCACCCCGGAAGAGATCC	ATCACGTCGTCATCCACAGG	234	60
Spo11	CGTGCTCATTCAGTGTTGACC	GGAGTACCCTTTCCCGTAACC	392	60
Sycp3	AGCATTCTGGGAAATCTGGGA	TCTGTACTTCACCTCCCACA	189	60
Apoptosis				
p53	GGAAACTTCTTCCTCCAGATGAT	TTTTGAGAAGGGACGGAAGAT	229	63
*Casp*3	GAGCTTGGAACGCGAAGAAA	CCATTGCGAGCTGACATTCC	224	60
Fas	GGCCCATTTTGCTGTCAACC	GTCTTCAAGTCCACACGAGGT	294	60
Steroidogenesis				
Star	ACTCACGTGGCTGCTCAGTA	AGTGGCTGGCGAACTCTATC	98	65
Cyp 11a1	ACTTCCGGTACTTGGGCTTT	CAGCACGTTGATGAGGAAGA	95	65
Cyp 17a1	GTCGTCAATCTCTGGGCACT	AGCTCCGAAGGGCAAGTAAC	135	65
Hsd3	ATATTGGAGGCCTGCGTCG	TCGGCCATCCTTTTGCTGTA	167	60

Testosterone Radioimmunoassay

To validate the results on the steroidogenesis genes expression level which was found modified homogeneously in the same direction (decreased overall) only for the GL exposed rats, we measured the intratesticular testosterone level by radioimmunoassay as previously described (Habert and Picon, 1984) for a subset of six adult rats exposed to G, V and GV during the gestational lactational period.

Data Analysis

Statistical tests were carried out using the BMDP statistical software (Dixon, 1988). All statistical comparisons between exposure groups were carried out by an analysis of variance (ANOVA). When the null hypothesis was rejected, post hoc Tukey tests using the variance from the experiment as a whole (for the considered variable) were used for pairwise comparisons between different exposures (as shown in the tables). Differences for qualitative variables between treated and control groups were tested using the Pearson chi-square test. The significance level was set at 0.05. However, for some aspects of the study, indicated as appropriate in tables and figures, a threshold at 0.10 was used due to the relatively small size of the groups.

Results

Developmental anomalies of the male reproductive tract in the GL (gestational/lactational period) exposed rats (F1) and their progeny (F2)

Reproductive malformations were observed sporadically in rats gestationally/lactationally exposed to genistein (G) alone or vinclozolin (V) alone or genistein/vinclozolin mixture (GV). Exposure to the mixture significantly induced developmental abnormalities in the urogenital tract in about half of cases ($p<0.002$), especially, undescended testis observed in one third of animals ($p<0.002$). The rate of developmental reproductive anomalies was not increased in the unexposed progeny (F2) whatever the exposure condition for the fathers. The relative AGD was significantly modified in the exposed rats and their progeny (Table 2).

Table 2. Relative anogenital distance in rats gestationally/lactationally exposed to genistein (G; 1 mg/kg bw/d) and/or vinclozolin (V; 1 mg/kg bw/d), and in F2 rats stemmed from exposed fathers

AGD/bw measured on PND21 on 50 rats per exposure group, by the same observer blind of exposure groups; ANOVA results and * $p<0.05$ and, ** $p<0.01$for paired comparisons between each exposure group and controls

	GL exposure				p-value
	Control	G	V	GV	
Exposure (F1)	0.273±0.016	0.268±0.019	0.290±0.027**	0.277±0.032**	0.001
Father exposure (F2)	0.365±0.033	0.375±0.028	0.384±0.027**	0.380±0.026**	0.001

Other reproductive endpoints at adulthood in the GL exposed rats (F1) and their progeny (F2)

The puberty onset was significantly increased in the vinclozolin and mixture exposure groups (Table 3). It was not significantly modified in the unexposed F2 (Table 3).

Table 3. Puberty onset (day of preputial separation) in rats gestationally/lactationally exposed (GL) to genistein (G; 1 mg/kg bw/d) and/or vinclozolin (V; 1 mg/kg bw/d), and in F2 rats stemmed from exposed fathers. $^{\dagger\dagger}p<0.05$; ‡not significant but tendency ($p<0.10$)

	GL exposure				p-value
	Control	G	V	GV	
Exposure (F1)	40.5±2.6 ††,‡	41.4±2.8	42.8±2.7 ††	42.5±1.9 ‡	0.02
Father exposure (F2)	39.3±1.3	40.6±1.0	39.9±1.3	40.2±1.0	0.17

The body weight and testis, epididymis, seminal vesicles and prostate relative weights were not found modified in the exposed rats as well as in their progeny (data not shown).

Fertility endpoints in the F1

Mating and fertility indexes were not modified whatever the type and period of exposure. Similarly, the litter size, mean pup weight, sex ratio at birth and rate of post implantation loss were unchanged in the exposed groups compared to the controls (data not shown).

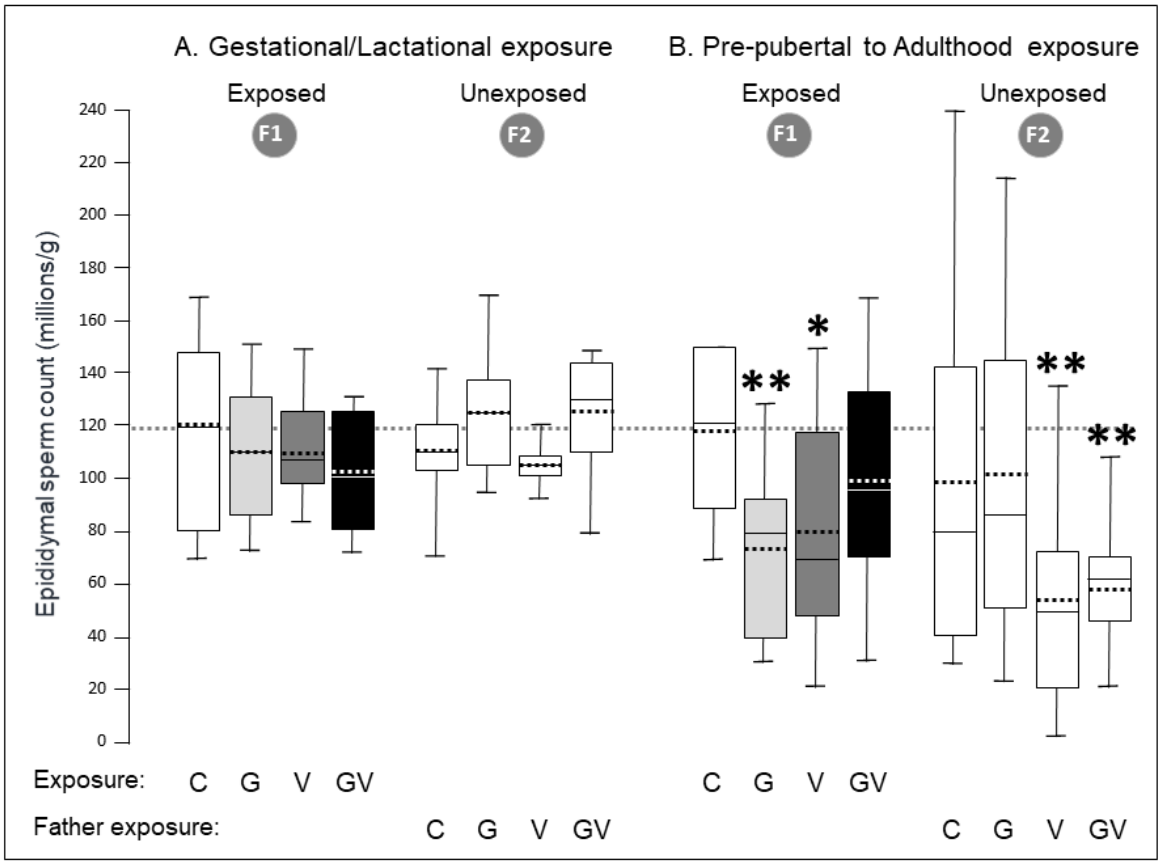
Reproductive endpoints in the PA (puberty to adulthood period) exposed rats (F1) and their progeny (F2)

The relative anogenital distance as well as putative developmental anomalies were not evaluated in the F1. The relative AGD was not significantly modified in the progeny. The puberty onset was not delayed in the three exposure groups neither in the progeny. There were no changes in the body weights and relative weights of the reproductive organs in the F1 and F2 except for the relative weight of epididymis that was significantly decreased for the animals exposed to genistein alone (F1; $p<0.05$). Similarly, no fertility endpoint was found modified in both F1 and F2 (data not shown).

Epididymal sperm number according to GL and PA exposure modalities (F1 and F2)

Neither each compound alone, nor their combination at the tested dose of 1mg/kg bw/d were found to affect the adult epididymal sperm count in both F1 and F2 when the exposure window was the gestational/lactational exposure (GL) (Fig. 2). In contrast, the PA exposure window for genistein or vinclozolin decreased the adult epididymal sperm number by almost half in comparison to the values in controls ($p<0.01$ and $p<0.05$, respectively). For the mixture GV, only a slight, not significant decrease was found. Finally, a significant decrease of the adult epididymal sperm count was founded in the progeny of the PA animals exposed to V and GV ($p<0.01$, for both) (Fig. 2)

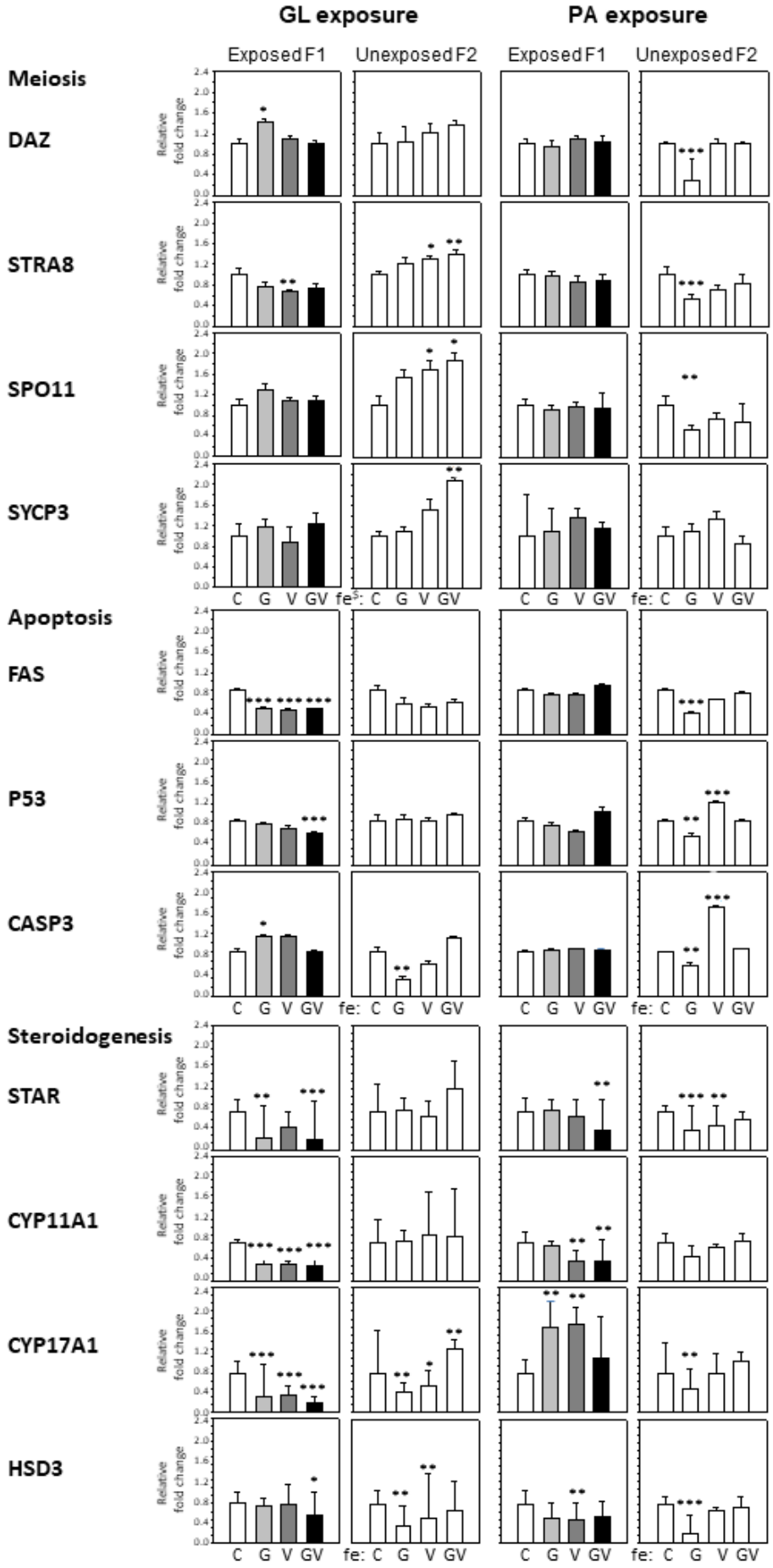
Fig. 2 Epididymal sperm counts according to the exposure modalities (see, Figure 1). Box plot displaying 10th, 25th, 50th, 75th, and 90th percentile values and the mean value (dotted line) of epididymal (cauda) sperm numbers in: A. Rats exposed to genistein (G) or vinclozolin (V) or a 1:1 mixture (GV) from gestation to lactation (GL) and their unexposed progeny and, B. Rats exposed to the same exposure modalities from pre-puberty to adulthood (PA) and their unexposed progeny. Measurements at PND100; n = 10 rats per group. The horizontal grey dotted line represents the mean value in the F1 control group (A. exposure modality) for reference. *: $p<0.05$ and **: $p<0.01$ compared to control by Tukey post-hoc test.



mRNA expression levels in F1 and F2 for GL and PA exposure windows

Figure 3 summarizes the mRNA expression levels in controls and exposed animals for a set of genes playing a major role in the processes of meiosis, apoptosis and steroidogenesis.

Fig. 3 mRNA expression levels (relative fold changes) in controls and exposed animals according to the exposure windows (GL: gestational/lactational; PA: pre-pubertal to adulthood) for a set of genes playing a major role in the processes of meiosis, apoptosis and steroidogenesis (n= 6 per group). *: $p<0.05$, **: $p<0.01$ and, $p<0.001$ compared to control by Tukey post-hoc test.



For both exposure window modalities the selected dose of 1mg/kg bw/d for G, V and their mixture had no or only a weak impact on the level of expression of the genes involved in the meiosis pathway tested in the exposed animals (F1). Only the

expression of *Daz* in G exposed animals was increased compared to the control group ($p<0.05$), and that of *Stra8* was found diminished for V ($p<0.01$). Interestingly, in the progeny of GL exposed fathers, the levels of expression of *Stra8*, *Spo11* and *Sycp3* were increased compared to the control group. Contrasting with these results, the levels of expression of *Daz*, *Stra 8* and *Spo11* in the progeny of the rats exposed to G during the PA period were lower than in the control group ($p<0.01$).

The PA exposure had no impact on the level of expression of the selected apoptosis genes. This contrasted with the impact of the GL exposure window (GL) that was found to modify (not as expected) the levels of expression of *Fas* for G, V and GV exposures, *p53* for GV and *Casp3* for G. Concerning the progeny of the rats exposed from gestation to lactation, only the expression of *Casp 3* was found reduced when compared to the controls ($p<0.01$). In contrast, the expression of *Fas*, *p53* and *Casp3* mRNAs levels for G was lower than the control group ($p<0.01$) and the expression levels of *p53* and *Casp 3* were increased for V compared to the control group ($p<0.001$) in the progeny of the rats exposed from the pre-pubertal period (PA).

The expressions of the genes of the steroidogenesis pathway were the most affected by both exposure windows, notably, the GL modality. For this exposure window, the expressions of *Star*, *Cyp11a1*, *Cyp17a1* for G and GV were significantly lower than the control ($p<0.01$), and the expression of *Cyp11a1* and *Cyp17a1* for V was also significantly decreased ($p<0.001$). *Hsd3* mRNA levels were significantly lower for GV compared to the controls ($p<0.05$). This was also observed for the progeny of the GL exposed rats (decreased expression levels of *Cyp17a1* and *Hsd3* for G and V; $p<0.05$ and $p<0.01$, respectively). The level of expression of crucial genes for the steroidogenesis pathway was also modified after the PA exposure modality, for example, the expressions of *Star* and *Cyp11a1* for GV, *Cyp11a1* and *Hsd3* for V were significantly lower than the control (all, $p<0.01$). The expression of the genes of the steroidogenesis pathway was also found modified in the progeny of the PA exposed rats, notably a decrease was observed for all the tested genes after exposure of the father to G.

Testosterone Radioimmunoassay

The intratesticular level of testosterone was significantly lower than the control only for the GV exposed rats (Supplementary Fig.).

Discussion

This study shows that an oral exposure to vinclozolin at a dosage below the NOAEL and/or to a realistic dosage of genistein impairs the male reproductive tract and function. It demonstrates clearly how the GL (gestational/lactational) and PA (pre-pubertal to adulthood) periods of exposure affect differently several reproductive endpoints. Particularly, it provides some substantial evidence that testis function is differentially affected according to the exposure windows studied, with modifications of the expression level of several genes playing a pivotal role in the processes of meiosis, apoptosis and steroidogenesis. In addition, we provide new evidence that exposure to such doses of endocrine active substances impacts the male reproductive tract in the progeny, though not directly exposed, and this in a different way depending on whether the fathers were exposed from gestation to lactation or from prepuberty to adulthood. It should be noted that due to the main

purpose and design of our study, we have not investigated here the crucial question of dose effect curves with EDs exposures, rather, we used a single (low) dose of genistein and vinclozolin previously shown to impact several male reproductive endpoints and consistent with human exposure levels (Eustache et al., 2009).

Only few and controversial data based on in vivo experiments concern the reproductive effects and potential modes of action of alimentary levels of genistein as well as dosages of vinclozolin around the NOAEL or less (for example, after a similar genistein exposure, the relative AGD was found either increased (Sherrill et al., 2010), or unmodified (Zhang et al., 2013) or, even decreased (Bobert et al., 2013)). Overall, we failed to find in the literature reports on the reproductive effects of genistein or vinclozolin with experimental designs similar to ours and most of them differed in many points (species, strain, modalities of exposure, number of animals, etc…), most being based on high dosages.

Here we did not find any marked effect of the 1mg/kg bw/d selected dosage of genistein on the set of classical reproductive endpoints studied for the GL exposure while the mRNA expression level of the genes of the steroidogenesis pathway was found to be lowered for this exposure window, the testicular level of testosterone being decreased but not significantly. The genistein exposure from the starting puberty only affected the epididymal weight and sperm count. This result is in accordance with Caceres et al. (2015) who reported a decreased sperm count after an oral administration of isoflavones in male Wistar rats during a PA period at both 0.56 mg/kg bw/d and 5.6 mg/kg bw/d. In contrast, the dosage of genistein used here was not found to affect the studied testicular mRNA expression levels overall for this exposure window. Regarding the effects of genistein on steroidogenesis, it is also known that exposure to genistein can affect testosterone levels during GL exposure (Akingbemi et al., 2007; Meena et al., 2017) but these studies used higher doses of genistein than ours. More precisely, Sherrill et al. (2010) have shown that a GL exposure to isoflavones (genistein and daidzein) may affect Leydig cell differentiation. Others studies have reported that in turn, isoflavones exposure decreases testosterone production (see for example, Svechnikov et al., 2005; Retana-Márquez S et al. 2016). However, the exact mechanisms of genistein action on testosterone production is not fully elucidated and it should be noted that others studies have not shown adverse effects on plasma testosterone levels whatever the doses of isoflavones used (genistein and daidzein) (Cederroth et al., 2010; Boberg et al., 2013).

In the present study, a GL exposure to 1mg/kg bw/d vinclozolin was unexpectedly found to increase the anogenital distance. This dosage was found to delay the puberty onset and decrease the mRNA expression levels of *Cyp11a1* and *Cyp17a1* and decrease (as a trend) the intratesticular testosterone level. The vinclozolin exposure from the starting puberty was essentially found to affect the epididymal sperm count. It is interesting to note that Elzeinova et al. (2008) using exposure modalities approaching ours have also reported a decreasing sperm count in mice exposed to 1 mg/ kg bw/d vinclozolin, during the PA window while the decrease was more pronounced for a GL period, a result contrasting with ours. Recently, Flick et al. (2017) for the same dosage in Wistar rats but using a chronical exposure reported no adverse reproductive effects.

For the doses tested here, the interpretation of the effects observed with the combination of genistein and vinclozolin is not obvious since, depending on the endpoints tested as previously discussed (Eustache et al., 2009) : i) an effect may be found with the mixture when each compound in isolation has no effect (for example, the rate of developmental anomalies or the level

of expression of *Hsd3* with GL exposure), ii) the effects observed with only one or both compounds separately are potentiated with the mixture (for example, the drop in intratesticular testosterone for GL exposure) or, iii) the effects with both compounds may be tempered (for example, the anogenital distance) or nullified (for example the sperm count for the PA exposure). It was previously reported that genistein at 1 mg/kg bw/d could antagonize the deleterious impact of a vinclozolin exposure at 1 mg/kg bw/d on the number of foetal/neonatal germ cells (Lehraiki et al., 2011). This last result and some of ours are consistent with the report of Zhang et al. (2013) showing that genistein may have a protective action against di (2-ethylhexyl) phthalate-induced reproductive effects.

Since our results with the GV mixture are preliminary and obtained with a study design not devoted to investigate the modes of action, further studies are desirable in order to know whether the results with the mixture reported here are reproducible. Overall and unfortunately, there is still a noticeable gap in our understanding of the mixture in vivo effects with low (realistic) dosages of endocrine active substances, which may act via different modes of action. This contrasts markedly with our knowledge in the field gathered thanks to powerful theoretical approaches devoted to mixtures (Kortenkamp, 2019). For example, it was evidenced that a mixture of high doses of antiandrogens has cumulative effects on androgen-sensitive endpoints in the pubertal male rat (Blystone et al., 2009; Christiansen et al. 2008). It was also demonstrated in vitro that a mixture of antiandrogenic pesticides has additive effects at very low concentrations (Orton et al., 2014).

It is well documented that foetus or new-born may be more vulnerable to EDs exposure compared to adults. However, the peripuberty years are also a critical period with the setting up of primary and secondary sexual characteristics as well as the initiation of spermatogenesis; of note, this period has been much less studied. A delayed puberty onset was observed after a GL exposure with vinclozolin and mixture. In parallel, a significant decrease in the expression of steroidogenesis genes (*Star*, *Cyp11a1* and *Cyp17a1*) was observed for rats exposed in utero to weaning with genistein and/or vinclozolin, and, the level of testosterone was decreased for the rats exposed during the gestation/lactation period to G, V and GV (only significant for the GV exposed group). In human, there is few data about effects of neonatal exposure to endocrine disruptors on pubertal timing in boys, and these data are sometimes contradictory. A decrease of testosterone and LH levels was reported in boys of mothers exposed in utero to polychlorinated biphenyls/polychlorodibenzofuranes (Hsu et al., 2005), PCB (Grandjean et al., 2012) and to DDT (Eskenasi et al., 2017). These data suggest a probable central origin of delayed puberty. In our study, when males were exposed in utero, we observed a delayed puberty onset and a decrease of intratesticular testosterone levels in adults. In a recent review (Parent et al., 2015), it was reported that a post weaning exposure to EDs (vinclozolin, DDE, DES) in male rodents leads to delayed puberty as opposed to an absence of effects after an in utero/neonatal exposure. The dose of EDs probably also plays a critical role (Parent et al., 2015). Indeed, Monosson et al (1999) and Yoshimura et al (2005) found that a PA vinclozolin exposure delayed pubertal maturation at 30 and 100 mg/kg bw/d. Thus, the pubertal effects of EDs exposures may depend on the dose but also on the period of exposure.

We failed to find in the literature reproductive studies focusing on EDs exposure windows allowing comparisons because of too different experimental schemes. We have previously reported the reproductive effects of an exposure to genistein and/or

vinclozolin from conception to adulthood using the dose of 1 mg/kg for each (Eustache et al., 2009). This previous study was carried out using the same animal facilities and experimental conditions than those of the present study allowing us to compare previous results to the results reported here. Table 4 summarizes the effects observed for the different exposure modalities.

Table 4. The impact of exposure windows, gestational-lactational *vs* pre-pubertal to adulthood *vs* conception to adulthood on various reproductive endpoints*

	Present study						Eustache et al., 2009		
	Gestational-lactational exposure			Pre-pubertal to adulthood exposure			Conception to adulthood exposure		
	G	V	GV	G	V	GV	G	V	GV
Developmental anomalies	-	-	↑**	ND	ND	ND	↑#	↑#	↑#
Relative AGD	-	↑	↑	ND	ND	ND	-	↓	↓
Puberty onset	-	↑	-	-	-	-	-	↑	↑
Body weight	-	-	-	-	-	-	↑	-	-
Relative weight of testis, epididymis, seminal vesicles and prostate	-	-	-	↓***	-	-	↓***	↓***	↓***;$
Epididymal sperm number	-	-	-	↓	↓	-	-	-	↓
Fertility endpoints	-	-	-	-	-	-	↓Litter size ↑ PIL	↑ PIL	↓Mating index ↓Litter ↑ PIL

* *From present study and Eustache et al., 2009, both studies being based on G: genistein exposure, V: vinclozolin exposure and, GV: mixture exposure at the same dose of 1mg/kg/d for both compounds; arrows represent significantly modified endpoints in exposed groups compared to control animals*

***cryptorchidism;* *** *only for epididymis;* # *macroscopically irregular, cystic, and/or enlarged epididymis;* $ *seminal vesicles*

PIL: Post implantation loss; ND: not determined.

Clearly, the higher number of disrupted male reproductive endpoints among which, decreased relative AGD, decreased sperm counts, impaired sperm motion parameters, decreased fertility, and increased post-implantation loss, was found for the exposure window encompassing all developmental steps (i.e., the exposure from conception to adulthood, comprising thus the

in utero, perinatal, pubertal and adult periods). This reinforces the concept that scrutinizing only the in utero or gestational/lactational periods cannot account for the effects that may arise under realistic conditions.

In the second generation, as for the parental exposure, no marked disruptions of the reproductive endpoints tested were observed except for the sperm count that was found significantly decreased for the rats whose fathers were exposed to vinclozolin and the mixture from the pre-pubertal period. It should be noted that the mRNAs of *p53* and *Casp*3 were overexpressed relative to control only for the offspring of vinclozolin exposed rats. Besides, the *Star* mRNA under-expression was also found for this group. Recently, EDs have been proved to cause multigenerational and transgenerational effects on both male and female rodent reproductive function (Anway et al., 2008; Chen et al., 2015; Zhou et al., 2017). When the generation F0 female is pregnant and exposed to an ED, then her offspring (F1 generation), are directly exposed to the ED in utero. The F2 generation is indirectly exposed via the germ cells of the F1 generation. Effects of ED observed in the F1 or F2 generations are considered to be multigenerational. The F3 generation is the first generation with no direct exposure to the ED, and effects would be considered transgenerational. In contrast, when F0 males are exposed to ED, effects in the produced F1 are considered multigenerational through exposure in the germline, and effects in the F2, the first generation not directly exposed, are considered transgenerational (Daxinger and Whitelaw, 2012; Lim and Brunet, 2013). The transgenerational effects of ED observed on male reproduction are for example: a shorter AGD (Manikkam et al., 2012), reduced fertility (Anway et al., 2005; Song et al., 2014), and decreased sperm production (Anway et al., 2005; Song et al., 2014; Doyle et al., 2013). These transgenerational effects are thought to be mediated *via* epigenetic mechanisms including DNA methylation, histone modifications and microRNA expression (Skinner, 2016; Larriba et al., 2016; Nilsson et al., 2018) though the modes of action are not fully elucidated. The vast majority of the transgenerational/multigenerational studies following EDs exposure have used wide dose ranges that are not always relevant to human exposure (for example, 100 mg/kg bw/d for vinclozolin; Anway et al., 2005) and exposure routes that are not comparable to daily human ones (i. e., intraperitoneal for vinclozolin; Doyle et al., 2013; Yuan et al., 2017). Here we provide evidence that an oral low dose exposure to EDs from the puberty may impact the reproductive function in the male progeny not directly exposed. The experimental design of our study does not consider a third generation. In addition the possible modes of action and the modest effects observed in the F2 remain to be confirmed. However, the present results indicate that exposure windows, dose ranges and mixtures of chemicals should be more considered in order to use animal studies as proxy for EDs human exposure.

Conclusion

We provide evidence that low dose exposure to endocrine active substances during the crucial developmental period starting from the prepuberty affects the adult testis function at least as much as an exposure during the GL period. This suggests better considering this developmental period in reproductive studies on environmental endocrine compounds. More studies based on relevant dose ranges, mixtures of chemicals, routes and exposure windows are required to better assess human exposure risk as well as for monitoring the possible reproductive effects in the subsequent generations or to better understand the mechanisms involved.

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Author Contributions

Edited the manuscript: Jacques Auger, Florence Eustache, Daniel Vaiman. Conceived and designed the experiments: Jacques Auger, Florence Eustache, Daniel Vaiman. Performed the experiments and analyses: Jacques Auger, Florence Eustache, Badria Bennani Smires, Delphine Moison, Raymond Berges, Marie-Chantal Canivenc Lavier. Analysed the data: Jacques Auger, Florence Eustache, Daniel Vaiman. Wrote the paper: Jacques Auger, Florence Eustache, Daniel Vaiman.

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