Endocrine disruptors *in utero* cause ovarian damages linked to endometriosis

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1. ABSTRACT

Timed pregnant Balb-C mice were treated from day 1 of gestation to 7 days after delivery with the endocrine disruptor bisphenol a (BPA) (100, or 1,000 μg/kg/day). After delivery, pups were hold for three months; then, ovaries were analyzed in their entirety. We found that in the ovaries of BPA-treated animals the number of primordial follicles and of developing follicles was significantly lower than in the untreated animals. Moreover, the number of atretic follicles was significantly higher in the treated animals. Finally, we found that the animals displaying endometriosis-like phenotype had a more severe impairment of the ovaries in term of number of primordial and developing follicles in comparison with the other mice exposed to BPA. In conclusion, we describe for the first time a complex phenotype in mice, elicited by pre-natal exposition to BPA, that includes ovarian lesions and endometriosis. Considering the high incidence of endometriosis and of the premature ovarian failure associated to infertility in these patients, the data showed prompt a thoroughly reconsideration of the pathological framing of these lesions.

2. INTRODUCTION

Numerous studies indicate that female reproduction efficiency has deteriorated over the past half century. A clear declining trend in conception rate has been registered, for example, in both Danish and US women (1,2). It is important to underline that some cultural changes in western countries (such as increased contraception or delayed childbearing) could have contributed to this phenomenon; however, it must be acknowledged the impact of environmental factors exposures to the foetus, mother, or father (3). Curiously, while the association between exposure to environmental factors and the reproductive capacity of males has been deeply investigated and has raised also public interest (4), a similar attention has not been dedicated to females. Nevertheless, the ability of synthetic chemicals to alter reproductive function and health in females has been described for the first time more than forty years ago, when the effects of diethylstilbestrol (DES) on the daughters of women given treatment with DES were firstly reported (5).

DES belongs to a group of synthetic chemical able to mimic hormones; these chemicals are called

endocrine disruptors. In detail, endocrine disruptors have been described as "exogenous chemical substances or mixtures that alter the structure or function (s) of the endocrine system and cause adverse effects at the level of the organism, its progeny, populations, or subpopulations of organisms, based on scientific principles, data, weight-of-evidence, and the precautionary principle" (6). Several experimental studies have reported that endocrine disruptors can affect at very low doses the endocrine system and the development of mammalian and non-mammalian species (7,8). Moreover, exposure to these environmental chemicals has been proposed to contribute to several gynaecologic pathologies, especially when exposures occur during critical periods of development (9-16). Nevertheless, it has been proposed in recent studies that hormonal perturbations during embryo-foetal or neonatal development may predispose individuals to numerous diseases and/or dysfunction later in life. These include increased incidence of tumours such as uterine adenocarcinomas (17) and breast cancers (18), obesity (19), hypertension and coronary disease (20). Most of these reported effects of endocrine disruptors in mammalians are caused through alteration of estrogen signalling, since it is crucial for proper ontogeny and function of all the female reproductive system (21).

Among environmental contaminants possessing hormone-like activity, Bisphenol A (BPA) is the firstly reported synthetic chemical causing selective oestrogen receptor modulation (22). The effects of this molecule have been deeply highlighted in recent years, since it is one of the highest volume chemicals produced worldwide, (23) and it accounts for the majority of the estrogenic activity that leaches from landfills into the surrounding ecosystem (24,25).

Our research group has recently demonstrated that pre-natal exposition to BPA is able to induce an endometriosis-like phenotype in mice (26). Endometriosis is a common gynaecological disease defined by the growth of endometrial glands and stroma outside the uterine cavity (27-29). The prevalence in the general female population is 6-10%; in women with pain, infertility or both, the frequency increases to 35-60% (30). Interestingly enough, there are several studies in humans linking exposition to endocrine disruptors with insurgence of endometriosis (3). In particular, a robust epidemiological study on a wide cohort of patients with endometriosis has shown that the rate of endometriosis is 80% greater among women exposed to DES *in utero* (32). Finally, it has been shown that severe ovarian damages occur in gonads operated on for ovarian endometriomas and this raises concerns about the role of surgery in endometriosis treatment (33,34).

With the aim to better establish the association between endocrine disruptors exposure and female reproductive health, with particular emphasis on the relationship between endometriosis and ovarian disruption, we decided to investigate the long-term effect of prenatal BPA exposure on the murine ovary genesis and to analyze these data in relation to the endometriosis-like phenotype induced by BPA.

3. MATERIALS AND METHODS

3.1. Animals and treatment

Adult female BALB-C mice were bred to male mice of the same strain in the animal facility at the Regina Elena Cancer Institute of Rome. Animals were maintained in temperature- and light-controlled (14L and 10D) conditions and had ad libitum water and food. Mice were fed Mouse Chow (Mucedola Srl, Milano, Italy) that tested negligble for estrogenic activity using the E-SCREEN assay (35), and tap water was supplied from glass bottles only. Cages and bedding also tested negative for estrogenic activity. The ethical committee of the Cancer Institute approved all the experimental protocols that were performed in accordance with Italian regulations (116/92) and with the Guide for the Care and Use of Laboratory Animals.

Vaginal plug detection was considered day 0 of pregnancy. Eighteen pregnant mice (6 per treatment group) were injected subcutaneously with 2% ethanol in physiological saline solution (control) or BPA (>99% purity; Sigma-Aldrich, Inc., St. Louis, MO) dissolved in ETOH 2% in physiological saline solution at a final concentration of 100 or 1000 μg/kg of body weight, on day 1 of gestation through the seventh day after delivery. Accordingly, henceforward the treated mice and their offspring will be identified as BPA-100 or BPA-1000, respectively.

To minimize any potential prenatal litter effects, all pups in a treatment group were pooled together, separated by sex, and then fostered (five female and five male pups per litter), to moms of the same treatment group. At 21 days of age, offspring were weaned, separated by sex, housed five per cage, and held without further treatment. At this time, moms were euthanized by carbon dioxide asphyxiation and tissues removed for further analyses. Female offspring were sacrificed at 3 months of age (n= 20 per treatment group). We had a total of sixty animals available for the experiments. Table 1 summarizes the number of animals used in this experimental setting.

3.2. Tissue collection and histology

Pelvic organs of the animals were collected en-block, fixed in paraformaldeyde and included in paraffin. We performed histological analysis, using Haematoxylin/Eosin and Haematoxylin/Van Gieson staining. Pelvic organs were analyzed in their entirety. To this end, four sections were taken every 100 microns and stained for histology. In detail, for the purpose of this work, all the sections representative of the ovarian tissues were selected and analyzed. Follicles were divided in three groups: primordial, developing follicles (including primary, secondary, tertiary and pre-antral) and atetric follicles. The number of follicles was calculated on two or three different representative sections of both ovaries of each animal and the median value was determined.

3.3. Statistical analysis

Statistical analyses were carried out using SPSS 10.0 for windows (SPSS Inc., Chicago, IL). For the

Table 1. The treatment groups of mice

Prenatal treatment	Number of treated dames	Number of female pups analyzed
Control	6	20
BPA-100	6	20
BPA-1000	6	20

Table 2. Median number of ovarian follicles found in the mice treated prenatally with BPA

Prenatal treatment	Primordial follicles	Developing follicles	Atretic follicles
Control	7,75	11,8	1,55
BPA-1000	3,8*	5,5*	3,3*
BPA-1000	2,65*^	4,5*°	3,4*

* Significantly different from the control (p=0.001). ^ Significantly different from BPA-100 (p=0.024). ° Significantly different from BPA-100 (p=0,035)

Table 3. Median number of ovarian follicles found in mice with endometriosis-like phenotype respect to all the other treated prenatally with BPA

Animals	Primordial follicles	Developing follicles	Atretic follicles
Endometriosis	2,63	4	3,32
No endometriosis	3,76*	5,90^	3,38

* Significantly different from group 1 (p=0.0023). ^ Significantly different from group 1 (p=0.001).

histological analysis, we compared lesion incidence in each hdose group with that in the control group using one-sided Fisher's exact tests; P-values < 0.05 were considered statistically significant. Experimental values were expressed as mean±SEM.

4. RESULTS

4.1. Effects on ovarian tissues caused by pre-natal exposure to BPA

Concerning the doses used, they were chosen since they were previously reported to be within the range of human exposures (36). Also, the route of administration, is considered relevant for assessing the potential for developmental effects of BPA in humans, since newborn mice do not demonstrate the rapid first pass metabolism of BPA as orally dosed adults do (37). No BPA was found in control animals; on the contrary, BPA was found in treated mice and female offspring. These data have been already described in a previous work of our research group (26).

There were no macroscopic defects observed in the reproductive tract of the animals studied. Histological analysis of the ovaries revealed a significant decrease in the number of primordial follicles in mice treated with BPA-1000 and BPA 100 respect to the controls (2 (C.I.95% 2,03-3,26) and 4 (C.I.95% 3,06-4,53) vs 8 (C.I.95% 7,09-8,40); p=0,0001), as wells as of all the developing follicles in the treated animals respect to the control (4 (C.I.95% 3,53-5,46) and 6 (C.I.95% 4,77-6,22) vs 12 (C.I.95% 10,66-12,93); p=0,0001). Moreover, the BPA-1000 animals, displayed a more severe phenotype respect to the BPA-100 mice, both in term of primordial follicles (p=0,024) and of developing follicles (p=0,035). Coherently, when we looked at the number of atretic follicles, we found that they were significantly more numerous in BPA-1000 and BPA-100 mice respect to the control (3 (C.I.95% 2,91-3,88) and 3,5 (C.I.95% 2,73-4,87) vs 1 (C.I.95% 1,22-1,87); p=0,0001). Interestingly, there was not a statistical significant difference in the number of atretic follicles in the two groups of treated animals (p=0,758). Table 2 summarizes the data presented. In

figure 1A and 1B, examples of the histological appearance of the ovaries analyzed are depicted.

4.2. Correlation between endometriosis-like phenotype and ovarian alterations

In a previous work on the same animals (26), we found in the adipose tissue surrounding the genital tracts of a consistent number of treated animals, endometriosis-like structures with the presence of both glands and stroma (30% in the BP-100 group and 35% in the BPA-1000 group). In order to investigate a possible relationship between endometriosis-like phenotype and ovarian alterations, we pooled together all the mice with endometriosis-like phenotype; then, we statistically compared the number of follicles found in these animals respect to all the other treated mice. Interestingly, we found that the mice with endometriosis-like phenotype had a significant lower number of primordial (2 (C.I.95% 2,04–3,21) vs 3,76 (C.I.95% 3,01–4,40); p=0023), and developing follicles (4 (C.I.95% 3,16-4,83) vs 6 (C.I.95% 5,21-6,59; p=0,001), while the number of atretic follicles was not significantly different (4 (C.I.95% 2,75-3,87) vs 3 (C.I.95% 2,87-3,88; p=0,851). Table 3 summarizes these data. In figure 1C, an example of the histological appearance of the ovaries of animals displaying endometriosis phenotype is depicted. Finally, we did not find any endometriosic lesion in all the ovaries analyzed.

5. DISCUSSION

The ability of endocrine disruptors to alter reproductive function and health in females are quite well characterized, thanks especially to the numerous works on the effects of DES exposition *in utero* (3). The mechanisms through which environmental factors, such as endocrine disruptors, can induce these alterations are still largely unknown, even if it is clear that female fetus is susceptible to environmentally induced reproductive abnormalities during a critical fetal exposure window (38). Three different pathways have been proposed to explain the influence of environment on the reproductive disorders. Firstly, a direct effect on gene expression induction. In fact, it has been demonstrated that xenoestrogen exposure alters
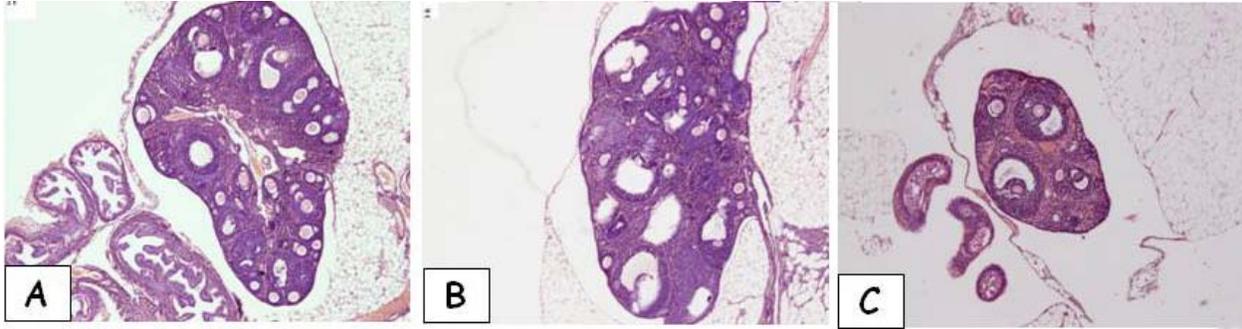
Figure 1. Histopathological appearance of the ovarian follicles in animals treated with BPA, respect to the control. A. Ovarian section from a control animal (Haematoxylin/Eosin, original magnification X4). B. Ovarian section from a BPA-1000 treated animal (Haematoxylin/Eosin, original magnification X4). C. Ovarian section from a BPA-1000 treated animal, displaying an endometriosis phenotype (Haematoxylin/Eosin, original magnification X4)

Hox gene expression in the developing mullerian system (39-41), and Hox genes are essential mediators of the correct axial development of the primitive Mullerian duct in the fallopian tubes, uterus, cervix and upper vagina (42). Secondly, an epigenetic mechanism through which endocrine disruptors alter transcriptional characteristics without changing DNA sequence (43). Thirdly, a neuroendocrine mechanism, where the nervous system registers the environment and send signals to the endocrine system. Nevertheless, at least for the central nervous system it is clearly demonstrated that a correct signalling from the environment is necessary for its accurate development (44).

Oocytes are the longest-lived, non-regenerating cells in the body, therefore are very good candidate to be subject to environmental exposures. The great majority of works on the effects of endocrine disruptors on ovaries has been performed in animals, because in humans these organs and their function are largely inaccessible. Nevertheless, numerous studies suggest that the female ovary is sensitive to interference by endocrine disruptors (45-47). Our work confirms these observations and further contributes to the characterization of the effects of *in utero* exposition to BPA. In particular, we have demonstrated a significant decrease in the number of primordial and developing follicles and an increase in the atretic follicles in the mice treated with BPA *in utero* respect to the control. This phenotype could account for some of the effects of endocrine disruptors on female reproductive disorders, especially that concerning with ovary-associated pathologies such as altered cyclicity and fecundability, polycystic ovary syndrome, and precocious menopause (3).

The absolutely original finding of this work is the demonstration that these ovarian alterations are strictly associated with the presence of an endometriosis-like phenotypes in the treated pups. Endometriosis is universally considered as a gynaecological disease caused by retrograde menstruation in the adult female, even if there is to date no definitive demonstration of this pathogenetic hypothesis (30). Our research group have recently demonstrated the presence of endometriosis-like structure in a consistent number of female human foetuses

analyzed at autopsy (48,49), thus suggesting a possible developmental origin of endometriosis (50). Nevertheless, the influences of endocrine disruptors on the correct development of the uterus are very well characterized (51), and the most straight observations in humans come again from the studies on women exposed *in utero* to DES (52,53)

Our data support the idea that pre-natal exposition to endocrine disruptors in a critical period of time can cause several reproductive disorders in the female fetus, being the ovary one of the first targets. The association between an endometriosis-like phenotype and a more acute disruption of the follicles, and the fact that we did not describe any case of ovarian endometriosis in our experimental model, suggest that the presence of endometriosis is an indirect evidence of a more severe disruption of the ovarian follicles, independently from the presence of endometriosic lesions in the ovarian parenchyma. This suggests, furthermore, an accurate investigation of ovaries in patients with endometriosis in order to evaluate ovarian efficacy of these patients. These experimental data, if confirmed, could significantly modify the therapeutic protocols actually performed for these pathological conditions.

Indeed, the observation that the effects on the ovarian follicles were variable and not all the treated animals displayed an endometriosis-like phenotype, further sustains the hypothesis of a complicated mechanism underlying the effects of endocrine disruptors, that includes genetic-epigenetic-neuroendocrine pathways (3). This could account for the variability we have detected in our experimental conditions.

In conclusion, we suggest that endometriosis, as well as ovarian disruption, should be included in the complex of disorders caused by *in utero* exposition to endocrine disruptors, that will affect the reproductive health performances during the adult life. We propose to frame all these congenital alterations of the female genital system in a syndrome, since they are caused by an identical pathogenetic mechanism. This syndrome of the female genital system, that would include endometriosis,

progressive ovarian failure, infertility, precocious menopause, associated with specific symptoms such as headache, pelvic cronic pain, dismenorrea, dispareunia, greater incidence of cancers, and many others, could be named syndrome of the ectopic endometrium. Considering the high incidence of endometriosis in humans and the enormous problems caused by precocious menopause with the associated infertility in humans endometriotic patients, these observations should be analyzed and possibly confirmed in greater experimental settings and the molecular basis carefully dissected.

6. ACKNOWLEDGMENTS

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