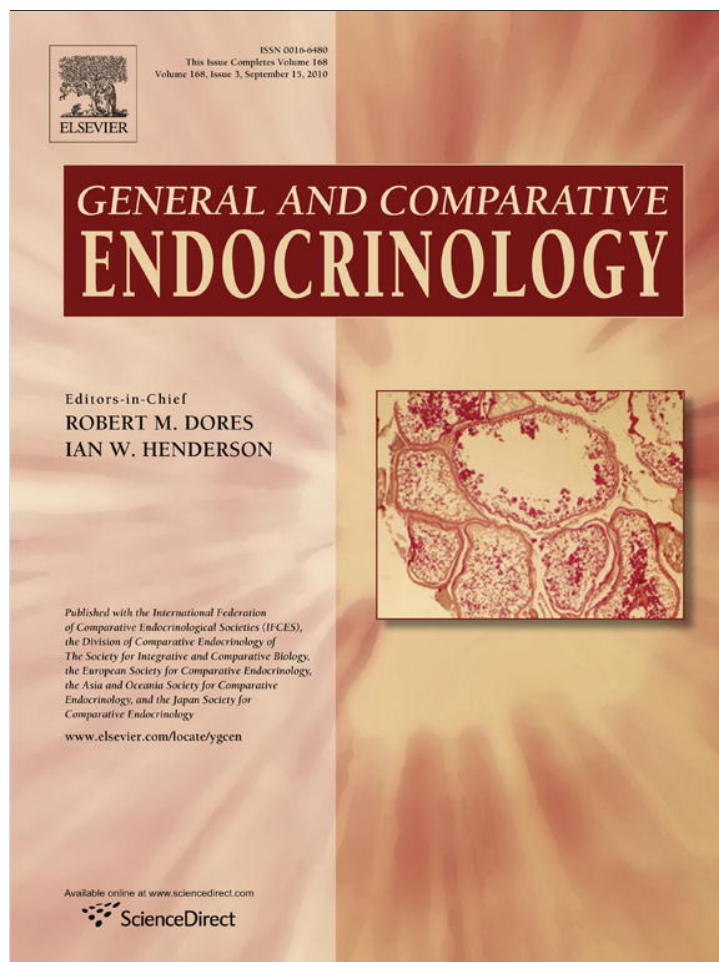




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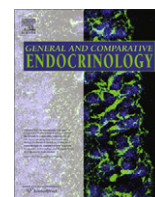
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Pre-natal exposure of mice to bisphenol A elicits an endometriosis-like phenotype in female offspring

Pietro G. Signorile ^{a,*}, Enrico P. Spugnini ^b, Luigi Mita ^{c,d}, Pasquale Mellone ^e, Alfredo D'Avino ^e, Mariangela Bianco ^c, Nadia Diano ^{c,d}, Lucia Caputo ^c, Francesca Rea ^a, Rosa Viceconte ^a, Marianna Portaccio ^{c,d}, Emanuela Viggiano ^{c,d}, Gennaro Citro ^b, Riccardo Pierantoni ^{c,d}, Vincenzo Sica ^{c,f}, Bruno Vincenzi ^g, Damiano G. Mita ^{c,d,h}, Feliciano Baldi ^e, Alfonso Baldi ^{a,c,e,**}

^a Fondazione Italiana Endometriosi, Rome, Italy

^b SAFU Department, Regina Elena Cancer Institute, Rome, Italy

^c Interuniversity Consortium INBB, Rome, Italy

^d Department of Experimental Medicine, Second University of Naples, Naples, Italy

^e Department of Biochemistry and Biophysics, Sect. Pathology, Second University of Naples, Naples, Italy

^f Department of General Pathology, Second University of Naples, Naples, Italy

^g Department of Oncology, Campus Biomedico University, Rome, Italy

^h Institute of Genetics and Biophysics of CNR, Naples, Italy

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ABSTRACT

Endometriosis is a chronic gynecological disease characterized by the growth of endometrial tissue outside the uterine cavity. Exposure to endocrine disruptors during critical period of development causes long-lasting effects, being the genital system one of the targets. This study describes the effects on female genital system caused by developmental exposure to the endocrine-disrupting chemical bisphenol A (BPA) during pre- and peri-natal development in mice. To this end, timed pregnant Balb-C mice were treated from day 1 of gestation to 7 days after delivery with BPA (100, or 1000 µg/kg/day). After delivery, pups were held for 3 months; then, pelvic organs were analyzed in their entirety and livers of both pups and moms were studied for the presence of BPA. We found in the adipose tissue surrounding the genital tracts of a consistent number of treated animals, endometriosis-like structure with the presence of both glands and stroma and expressing both estrogen receptor and HOXA-10. Moreover, cystic ovaries, adenomatous hyperplasia with cystic endometrial hyperplasia and atypical hyperplasia were significantly more frequent in treated animals respect to the controls. Finally, BPA was found in the livers of exposed moms and female offspring. In conclusion, we describe for the first time an endometriosis-like phenotype in mice, elicited by pre-natal exposition to BPA. This observation may induce to thoroughly reconsider the pathogenesis and treatment of endometriosis, considering the high incidence of endometriosis and the problems caused by associated infertility.

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1. Introduction

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” (EPA, 1998).

* Correspondence to: Pietro G. Signorile, Fondazione Italiana Endometriosi, Via E. Longoni, 81, 00159 Rome, Italy. Fax: +39062255261.

** Corresponding author at: Fondazione Italiana Endometriosi, Via E. Longoni, 81, 00159 Rome, Italy. Fax: +39062255261.

E-mail addresses: alfonsobaldi@tiscali.it, research@endometriosi.it (A. Baldi).

Among environmental contaminants possessing hormone-like activity (endocrine disruptors), Bisphenol A (BPA) is the firstly reported synthetic chemical causing selective estrogen receptor modulation (Doods and Lawson, 1936). The effects of this molecule have been deeply highlighted in recent years, since it is one of the highest volume chemicals produced worldwide, (Burridge, 2003) and it accounts for the majority of the estrogenic activity that leaches from landfills into the surrounding ecosystem (Kawagoshi et al., 2003; Coors et al., 2003). In fact, it is used in the manufacture of polycarbonate plastic, the resin that lines most food and beverage cans, dental sealants, and as an additive in other plastics, such as PVC (Maffini et al., 2006). Therefore, it is strongly suggested that mammals and humans are routinely exposed to this compound. For this reason its bioremediation (Diano et al., 2007) and

bio-determination (Mita et al., 2007) in polluted waters and some molecular mechanisms by which it interacts at cellular level, have been intensively investigated in last years (Bolli et al., 2008; Ricupito et al., 2009; Bontempo et al., 2009).

Several experimental studies have reported that endocrine disruptors can affect at very low doses the endocrine system and the development of mammalian (humans included) and non-mammalian species (Tyler et al., 1998; McLachlan, 2001; Bompadre et al., 2001; Foster, 2008a). In particular, an increasing number of “low-dose” studies in mammals have implicated perinatal BPA exposure in a variety of abnormalities in the female reproductive tract, including early onset of vaginal opening (Honma et al., 2002), early onset of puberty (Howdeshell et al., 1999), altered estrus cyclicity (Markey et al., 2005), altered plasma levels of luteinizing hormone (Rubin et al., 2001), altered vaginal, ovarian and uterine histology (Schonfelder et al., 2004; Newbold et al., 2007a, 2009), and in neocortical development (Nakamura et al., 2007). Nevertheless, increasing experimental evidences are showing that exposure to toxicants during critical periods of pre- and peri-natal development can have long-lasting effects. In particular, it has been proposed in recent studies that hormonal perturbations during embryo-fetal or neonatal development may predispose individuals to numerous diseases and/or dysfunction later in life. These include reproductive problems (Newbold, 2009), increased incidence of tumors such as uterine adenocarcinomas (Newbold et al., 2001) and breast cancers (Davids et al., 1993), obesity (Newbold et al., 2007b), hypertension and coronary disease (Sallout and Walker, 2003). In this respect, an genetic–epigenetic reprogramming may occur (Prins, 2008).

Endometriosis is a common gynecological disease defined by the growth of endometrial glands and stroma outside the uterine cavity (Giudice and Kao, 2004; Baldi et al., 2008). The prevalence in the general female population is 6–10%; in women with pain, infertility or both, the frequency increases to 35–60% (Bulun, 2009). Endometriosis is usually accompanied by infertility, chronic pelvic pain and adhesion formation. Deep infiltrating endometriosis of recto-vaginal septum is a particular form of endometriosis located under the peritoneal surface (Signorile et al., 2009a, b). These kind of lesions biologically are very active and are strongly associated with pelvic pain symptoms. Despite the high morbidity and health care cost associated with endometriosis, research scientists remain unsure as to the definitive cause(s) of this disease. Currently, none of the pathogenetic theories proposed, such as retrograde menstruation implants, coelomic metaplasia or staminal cells hypotheses, has definitively been proved (Bulun, 2009).

It is well established that endometriosis is influenced by steroid hormones. Indeed, endometriosis lesions have estrogen and androgen receptors and estrogens can promote their growth (Witz, 1999). Thus, it is believed that conditions that alter steroid hormone levels may influence the growth and survival of endometriosis plaques. In particular, there is increasing interest in the impact of the exposure of the female reproductive tract during development to environmental chemicals with estrogenic activity, the so-called endocrine disruptors.

Interestingly enough, there are several studies in humans linking exposition to endocrine disruptors with insurgence of endometriosis (Foster, 2008b). 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 the endocrine disruptor diethylstilbestrol in utero (Missmer et al., 2004). Our research group has recently demonstrated the presence of ectopic endometrium in 11% of human fetuses analyzed during autopsy (Signorile et al., 2009b). This observation has been the first direct and systematic demonstration of the theory of developmentally misplaced endometrial tissue as the cause of endometriosis. Leaving from this background, we decided to

investigate the long-term effect of pre-natal BPA exposure on the murine female reproductive tract with particular emphasis on endometriosis.

2. Materials and methods

2.1. 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 negligible for estrogenic activity using the E-SCREEN assay (Markey et al., 2005), 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. Concerning the doses used, they were chosen since they were previously reported to be within the range of human exposures. Also, the route of administration, namely subcutaneous injections, 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 (Volkel et al., 2008). Concerning the direct consequence on human health, it should be noted that the BPA doses were low and within the range of human exposure (NTP, 2008).

To minimize any potential pre-natal 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 (see below). 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 that is twenty mice for each treatment group.

2.2. Tissue collection and histology

Pelvic organs of the animals were collected en-block, fixed in paraformaldehyde 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 µ and stained for histology. If a microscopic lesion was observed, additional serial sections were made to include the entire area of pathological change and to perform immunohistochemistry.

2.3. Immunohistochemistry

For immunohistochemistry 5–7 µm specimen sections embedded in paraffin, were cut, mounted on glass and dried overnight at 37 °C. All sections were then deparaffinized in xylene, rehy-

drated through a graded alcohol series and washed in phosphate-buffered saline (PBS). PBS was used for all subsequent washes and for antiserum dilution. Tissue sections were quenched sequentially in 3% hydrogen peroxide in aqueous solution and blocked with PBS-6% non-fat dry milk (Biorad, Hercules, CA, USA) for 1 h at room temperature. Slides were then incubated at 4 °C overnight at 1:100 dilution with the following antibodies: the affinity-purified rabbit antibody ER α for the estrogen receptor, and the goat polyclonal HOXA-10 (Santa Cruz, Santa Cruz, CA, USA). After three washes in PBS to remove the excess of antiserum, the slides were incubated with secondary biotinylated antibodies (Vector Laboratories, Burlingame, CA, USA) at 1:200 dilution in PBS-3% non-fat dry milk (Biorad, Milan, Italy) for 1 h. All the slides were then processed by the ABC method (Vector Laboratories, Burlingame, CA, U.S.A.) for 30 min at room temperature. Diaminobenzidine (Vector Laboratories) was used as the final chromogen and haematoxylin was used as the nuclear counterstaining. Negative controls for each tissue section were prepared by leaving out the primary antiserum. Positive controls of tumor tissues expressing each of the antigens analyzed were run at the same time. All samples were processed under the same conditions.

2.4. Samples for BPA detection

In order to assess the presence of BPA in moms, as well as in female offspring, liver was explanted, immediately frozen in dry ice and stored at –80 °C in glass containers. Liver was chosen since it represents the most metabolizing tissue. The liver samples were kept at –80 °C and thawed before the high performance liquid chromatography (HPLC) analysis.

2.5. Methodology for BPA detection

All reagents were purchased from Sigma (Sigma–Aldrich, Inc., St. Louis, MO). Glass containers were used for sample treatment and their storage. For sample analysis two preparation steps were required: homogenization and extraction. The homogenization of the tissues, previously thawed, was performed by adding methanol to the samples and using *Diax 600* homogenizer. The choice of methanol is due to BPA hydrophobicity. After 45 min of mechanical agitation, samples were centrifuged to obtain two phases, with the organic one (supernatant) containing the molecules of interest. The supernatant was recovered, filtered with sterile gauze and dried with a stream of nitrogen. Residuals were re-suspended in acetone/hexane (3:97). Solid phase extraction was performed using Sep-Pak Light Florisil cartridge (Waters, WAT023543). Elution of BPA was performed with Acetone/Hexane (20:80). The HPLC system (Varian Prostar) was equipped with a UV–vis and a fluorescence detector. The BPA was detected at 220 and 280 nm. A microorb 300-C18 column (250 $\times$ 4.6 cm, 5 mm) was used for reverse-phase separations with a 20 μ L sample loop. The mobile phase was prepared by mixing acetonitrile/water (30:70) in the gradient mode, at a flow rate of 1 mL/min. BPA is eluted at 11 min. From a calibration curve the BPA concentration was calculated taking in account the peak area. Occasionally the BPA presence in tissues has been validated by GC–MS technique. Results are presented as average value of the total samples analyzed for each animal group and are expressed as ng of BPA for mg of tissue.

2.6. Statistical analysis

Statistical analyses were carried out using SPSS 10.0 for windows (SPSS Inc., Chicago, IL). For the histological analysis, we compared lesion incidence in each dose group with that in the control group using one-sided Fisher's exact tests; *P*-values < 0.05 were considered statistically significant. OP concentration in the liver

of treated or control groups was analyzed by two-way ANOVA, considering the factors treatment (BPA 100 μ g/kg of body weight, BPA 1000 μ g/kg of body weight, ETOH 2%) and age (adult mice, female offspring). Significance was recognized for *P* < 0.05. Multiple comparisons were performed with the Fisher's least significant difference (LSD) post hoc test. Experimental values were expressed as mean $\pm$ SEM.

3. Results

There were no macroscopic defects observed in the reproductive tract of the animals studied. Histological analysis revealed cystic ovaries (Fig. 1A), after 3 months, which were more common in animals from both BPA treated groups, compared to the controls (10% in Controls, 45% in BPA-100 and 50% in BPA-1000). These differences were statistically significant by Fisher's exact tests (*P* = 0.008). A comparison of ovarian and uterine abnormalities in Control and BPA treated mice is shown in Table 1. We found no statistical difference in the number of mice without corpora lutea in any group (data not shown). Concerning the uterine abnormalities, we observed adenomatous hyperplasia with cystic endometrial hyperplasia (Fig. 1B) in BPA-100 (25%) and BPA-1000 groups (25%), while the incidence was lower in the Control group (10%). Furthermore, we observed in the treated animals also some cases of atypical hyperplasia (Fig. 1C and D), which is considered a precursor lesion of the estrogen-associated uterine adenocarcinoma, while we did not find any case in the control group. This difference had a significant trend, when analyzed by Fisher's exact tests (*P* = 0.085).

When we analyzed the pelvic organs in their entirety, interestingly we found in the adipose tissue surrounding the genital tracts of a consistent number of treated animals, endometriosis-like structure with the presence of both glands and stroma. The incidence was of 30% in the BP-100 group and 35% in the BPA-1000 group, while we found only one case in the control. This different incidence was statistically significant (*P* = 0.024). The glands had an overtly endometrial appearance, identical to eutopic endometrium, while the stroma typically resembled eutopic inactive or proliferative endometrial stroma, including the presence of a network of small arterioles. Some examples of these endometriosis-like structures are depicted in Figs 2 and 3. In order to support the endometrial nature of these structures, we performed immunohistochemical experiments to analyze estrogen receptor and HOXA-10 expression, proteins that are well known to be expressed in the female genital tract. As shown in Fig. 4, the endometriosis-like structure expressed both estrogen receptor and HOXA-10 in the nucleus.

Finally, we looked at the presence of BPA in the tissues of the animals. No BPA was found in control animals, either adult mice or female offspring. On the contrary, BPA was found in treated mice and female offspring (Fig. 5). The average BPA concentration in adult female mice in the BP-100 treatment group was 0.74 ± 0.13 ng BPA/g of tissue, while in the BP-1000 treatment group it was 1.64 ± 0.53 ng BPA/g of tissue. The average BPA concentration in female offspring in the BP-100 group was 0.26 ± 0.06 ng BPA/g of tissue, while in the BP-1000 treatment group it was 0.24 ± 0.06 ng BPA/g of tissue. The analysis of variance showed significant effects for age [*F*(1, 65) = 45.6750, *P* < 0.01], for treatment [*F*(2, 65) = 38.879, *P* < 0.01], and for the age-treatment interaction [*F*(2, 65) = 23.066, *P* < 0.01]. The LSD post hoc test showed a significant BPA concentration in the liver of the BP-100 group of moms as compared with control group (*P* < 0.05) and female offspring of treated pregnant mice (*P* < 0.05). Furthermore, a significant BPA concentration was found in the liver of the BP-1000 group of adult mice as compared with the control group (*P* < 0.01) and female offspring of treated mice (*P* < 0.01). A significant BPA

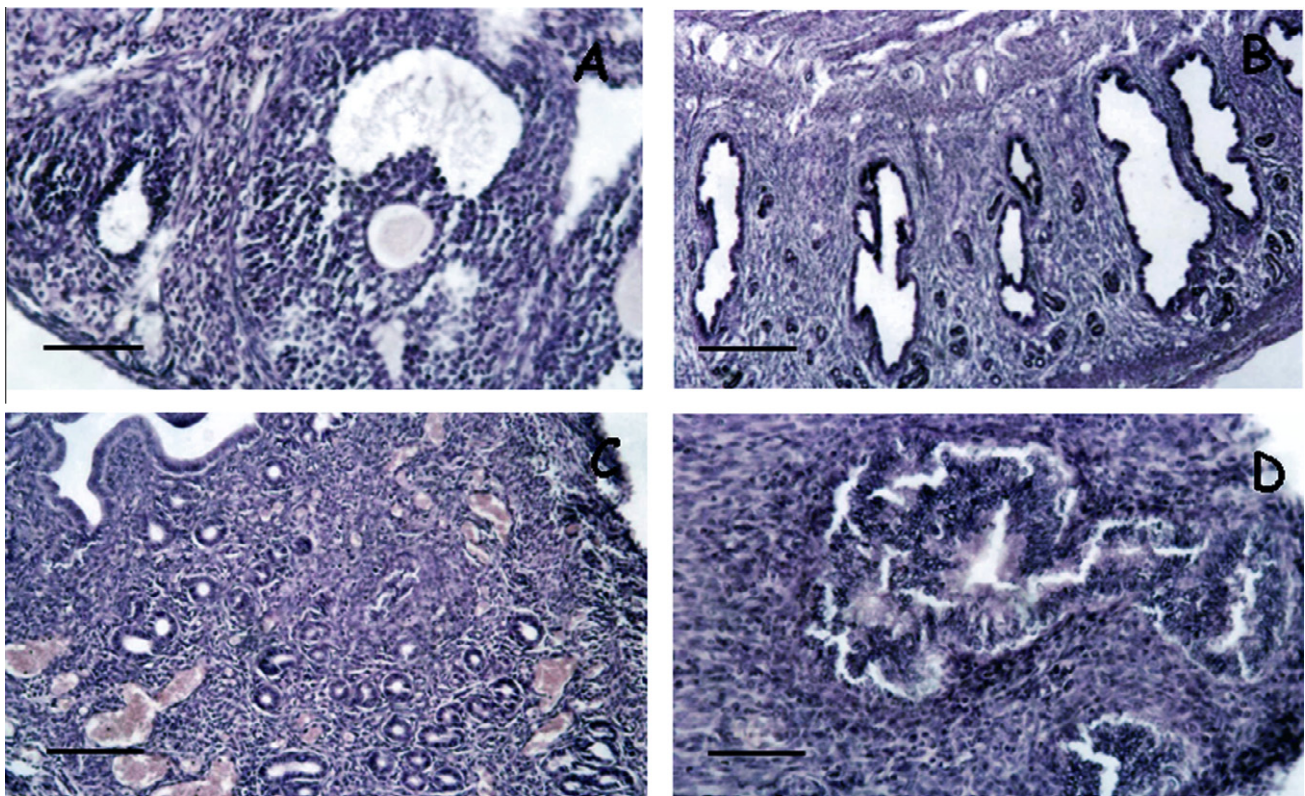


Fig. 1. Histopathological characteristics of the abnormalities found in mice treated pre-natally with BPA (scale bar = 100 μ). PANEL A cystic ovary (haematoxylin/eosin, original magnification 40 $\times$). PANEL B adenomatous hyperplasia of the endometrium (haematoxylin/eosin, original magnification 20 $\times$). PANEL C atypical hyperplasia of the endometrium: note the irregularly shaped glands with little intervening stroma (haematoxylin/eosin, original magnification 20 $\times$). PANEL D higher magnification showing “piling up” of cells with hyper-chromatic nuclei and some mitotic figures (haematoxylin/eosin, original magnification 40 $\times$).

Table 1
Abnormalities found in the mice treated pre-natally with bisphenol A (BPA).

Pre-natal treatment	Ovaric cysts	ADH	ATH	Endometriosis-like
Control	2/20 (10%)	2 (10%)	0/20 (0%)	1/20 (5%)
BPA-100	9/20 (45%)*	5 (50%)	3/20 (15%)	6/20 (30%)**
BPA-1000	10/20 (50%)*	10/20 (50%)*	4/20 (20%)	7/20 (35%)*

ADH = adenomatous hyperplasia.

ATH = atypical hyperplasia.

* Significantly different from the control ($P = 0.008$).

** Significantly different from the control ($P = 0.024$).

concentration in the liver of the BP-1000 group of mice compared to the BP-100 group of moms ($P < 0.05$) was also detected. There was a significant increase of BPA accumulation in the liver of the BP-100 and BP-1000 female offspring compared to the control group ($P < 0.05$). No significant difference was evaluated for the BP-100 and BP-1000 female offspring.

4. Discussion

The absolutely original finding of this work is the demonstration of endometriosis-like structures in the adipose tissue surrounding the genital tract of a significant number of treated pups. Endometriosis is universally considered as a gynecological disease caused by retrograde menstruation in the adult female, even if there is to date no definitive demonstration of this pathogenetic hypothesis (Bulun, 2009). Recent data from our group, have shown the presence of endometriosis-like structure in a consistent number of female human fetuses analyzed at autopsy (Signorile

et al., 2009b, 2010) thus suggesting a possible developmental origin of endometriosis. Indeed, strong epidemiological data link the in utero exposure to DES and the insurgence of endometriosis later in the adult life (Missmer et al., 2004).

The effects on reproductive tract development caused by pre- and/or peri-natal exposure to BPA are well characterized. In utero exposure appears to have an effect on the uterus that persists in the adult (Markey et al., 2005). Several *in vitro* and *in vivo* experiments have highlighted the molecular mechanisms through which endocrine disruptors are able to interfere with normal development of the reproductive tract. In fact, it has been demonstrated that xeno-estrogen exposure alters Hox gene expression in the developing müllerian system (Block et al., 2000; Smith and Taylor, 2007; Suzuki et al., 2007; Taylor, 2008). Hox genes are essential mediators of the correct axial development of the primitive Müllerian duct in the fallopian tubes, uterus, cervix, and upper vagina (Du and Taylor, 2004); they are highly evolutionarily conserved transcription factors and are expressed in a temporally and spatially linear manner (Taylor et al., 1997).

In our experimental model of exposure to BPA during the embryo-fetal life, we find in the genital tract of the treated pups some of the histopathological abnormalities described in previous works dealing with similar experimental procedures, such as ovarian cysts and cystic endometrial hyperplasia. (Schonfelder et al., 2004; Nayyar et al., 2007; Newbold et al., 2007a, 2009). Interestingly, the incidence of pre-malignant lesions, that is atypical endometrial hyperplasia, was also described, and this is in accordance with the recent data on the potentially carcinogenic alterations in female reproductive tissues caused by low pre-natal doses of BPA (Newbold et al., 2009). The reason why we did not find any case of malignancy must be ascribed to the fact that animals in

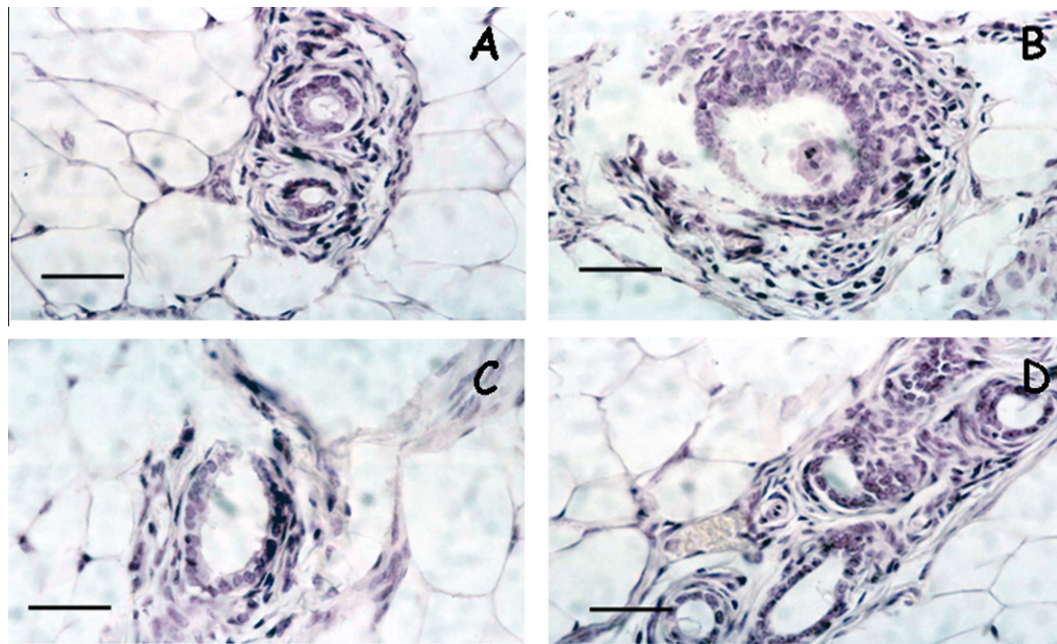


Fig. 2. Histopathological appearance of micronodular endometriosis-like structures found in the adipose tissue surrounding the genital organs of the mice treated pre-natally with BPA (scale bar = 100 μ). **PANEL A** two endometriotic-like glands delimited by stromal cells (haematoxylin/eosin, original magnification 40 $\times$), found in the adipose tissue surrounding the uterus. **PANEL B** a single endometriotic-like gland (haematoxylin/eosin, original magnification 40 $\times$). **PANEL C** another single endometriotic-like gland surrounded by stromal cells (haematoxylin/eosin, original magnification 40 $\times$). **PANEL D** micronodular endometriosis-like with four small glands visible in the context of a network of small arterioles (haematoxylin/eosin, original magnification 40 $\times$).

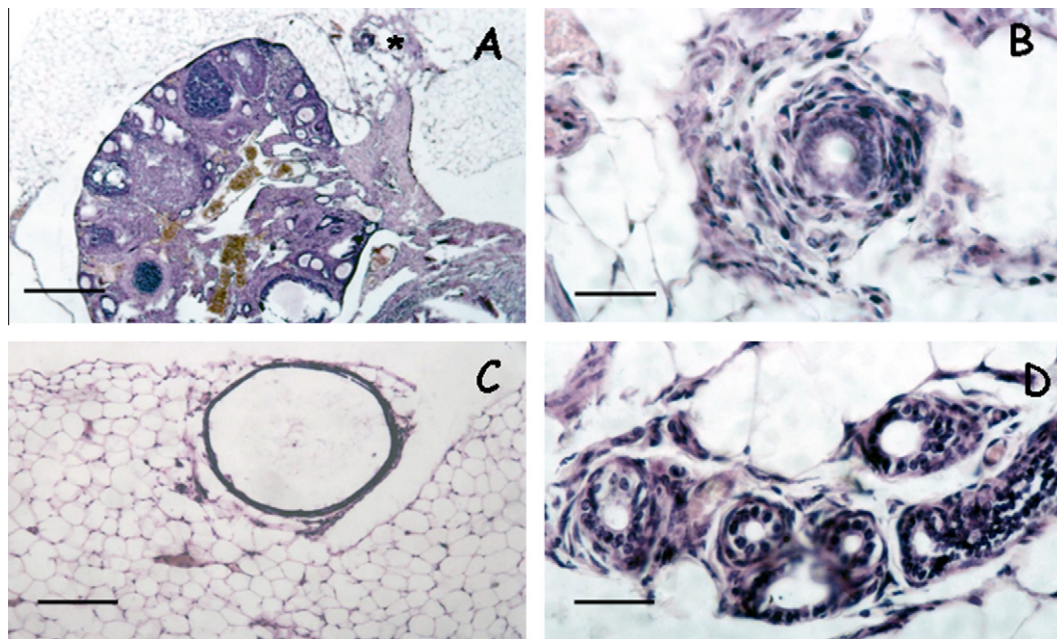


Fig. 3. Further examples of endometriosis-like structures found in the adipose tissue surrounding the genital organs of the mice treated pre-natally with BPA (scale bar = 100 μ). **PANEL A** at low-power magnification an endometriosis-like gland (indicated by an asterisk) is visible close to the ovary of a treated animal (haematoxylin/eosin, original magnification 10 $\times$). **PANEL B** higher magnification of figure in panel A, better showing the histopathological appearance of the endometriotic-like gland with a thin cuff of peri-glandular endometriotic stroma. (Haematoxylin/eosin, original magnification 40 $\times$). **PANEL C** a single endometriotic-like cyst lined by epithelium and only focally surrounded by stromal cells (haematoxylin/eosin, original magnification 20 $\times$). **PANEL D** multiple endometriotic-like glands in the context of a network of small arterioles (haematoxylin/eosin, original magnification 40 $\times$).

our experimental setting have been sacrificed at 3 months of age and probably they had not the time to fully develop tumors of the genital tract. The morphological and immunohistochemical characteristics of the endometriosis-like structure described in our experimental model are exactly identical to that of the normal endometrium and stroma present in the uterus of the same ani-

mals, as demonstrated by immunohistochemical expression of estrogen receptor and HOXA-10. Nevertheless, the mouse is a not menstruating animal, therefore the retrograde menstruation theory can not apply to this case. Interestingly, we found an endometriosis-like phenotype also in a single animal of the Control group. This observation sustains the fact that naturally occurring endome-

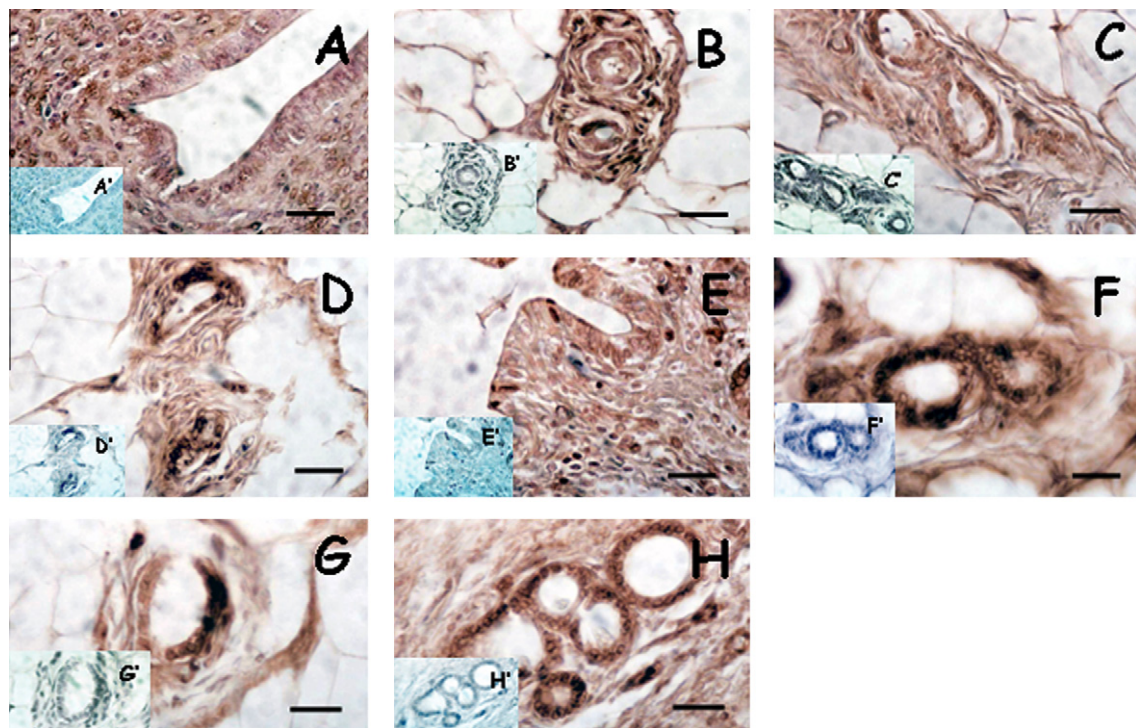


Fig. 4. Estrogen receptor and HOXA-10 expression in the uterus and in the endometriosis-like structures of the treated animals (scale bar = 100 μ). PANEL A estrogen receptor nuclear expression in the endometrium and in the stromal cells of the uterus of the analyzed animals. The negative control is shown in the inset. (ABC original magnification 20 $\times$). PANEL B a micronodular endometriosis-like structure with two visible glands, showing clear nuclear staining for estrogen receptor. The negative control is shown in the inset. (ABC, original magnification 40 $\times$). PANEL C two endometriotic-like glands in the adipose tissue surrounding the genital tract of a treated animal, displaying estrogen receptor expression in their nuclei. The negative control is shown in the inset. (ABC, original magnification 40 $\times$). PANEL D further example of endometriotic-like glands expressing estrogen receptor in their nuclei. The negative control is shown in the inset. (ABC, original magnification 40 $\times$). PANEL E HOXA-10 nuclear expression in the endometrium and in the stromal cells of the uterus of the analyzed animals. The negative control is shown in the inset. (ABC original magnification 20 $\times$). PANEL F a micronodular endometriosis-like structure with two visible glands, showing clear nuclear staining for HOXA-10. The negative control is shown in the inset. (ABC, original magnification 40 $\times$). PANEL G a single endometriotic-like gland in the adipose tissue surrounding the genital tract of a treated animal, displaying HOXA-10 expression in its nuclei. The negative control is shown in the inset. (ABC, original magnification 40 $\times$). PANEL H further example of endometriotic-like glands expressing HOXA-10 in their nuclei. The negative control is shown in the inset. (ABC, original magnification 40 $\times$).

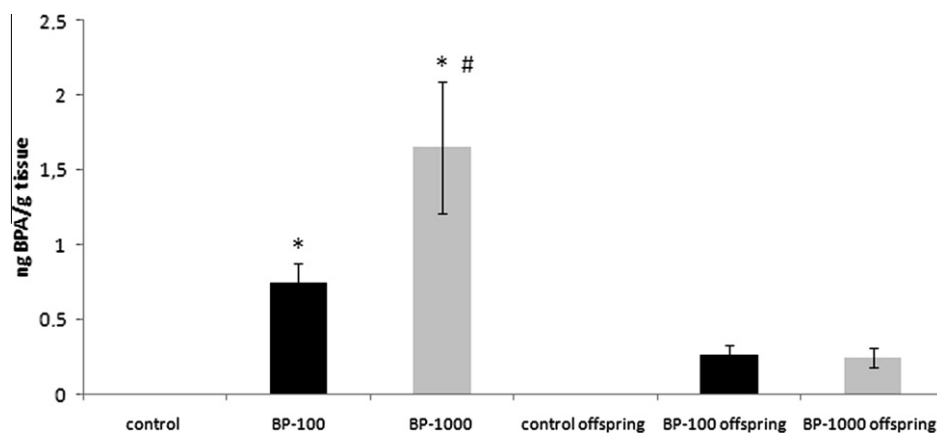


Fig. 5. Mean $\pm$ SEM of BPA concentration in the liver of the mice injected with 100 μ g (BP-100) or 1000 μ g (BP-1000) of BPA/kg of body weight and the female offspring of BP-100 (BP-100 offspring) or BP-1000 (BP-1000 offspring). * $P < 0.05$ BP-100 group versus control groups and BP-100 offspring or BP-1000 offspring, and BP-1000 versus BP-100. # $P < 0.01$ BP-1000 versus control groups and BP-100 offspring or BP-1000 offspring.

triosis should not be confined only to menstruating animals, and that endometriosis is a disease not necessarily correlated to the presence of endocrine disruptors, that in any case could operate synergistically. Nevertheless, the presence in humans of the disease in early puberty and exceptionally also in newborns (Batt and Mitwally, 2003; Diez Garcia et al., 1996; Marsh and Lauferm, 2005; Ebert et al., 2009), as well as in women affected by the

Mayer-Rokitansky-Küster-Hauser, a syndrome characterized by congenital aplasia of the uterus and the upper part of the vagina (Balci et al., 2008; Mok-Lin et al., 2009), and the presence of endometriosis in such remote areas as the lungs, skin, lymph nodes, breasts (Bulun, 2009; Baldi et al., 2008), sustains the fact that naturally occurring endometriosis can not be considered necessarily correlated to the menstruation.

Our data support the idea that endometriosis should be considered a developmental disease, caused by alteration in the correct axial development of the Mullerian system during a critical period of embryogenesis by changes in genetic–epigenetic programming. Indeed, the observation that not all the treated animals display an endometriosis-like phenotype, suggest that the exposition to endocrine disruptors during a critical window of pre-natal development (Selevan et al., 2000) might induce in genetically predisposed animals the insurgence of endometriosis. The exact definition of this critical window for exposure during the pre-natal development, as well as of the genetic background indispensable for the insurgence of the phenotype, will help in the comprehension of the pathogenesis and progression of endometriosis. The circumstance that the BPA concentration was found in the offspring of exposed mice strengthen the possibility of a relationship between exposure to BPA and endometriosis occurrence, confirming previous data showing that BPA is efficiently absorbed and distributed to the whole organism, including the reproductive tract and fetuses (Shin et al., 2002; Cabaton et al., 2006). Interestingly, there were no significant differences in the degree of the endometriosis-like phenotype when the two BPA doses (100 and 1000 ug/kg) are compared. This is consistent, however, with the fact that the BPA levels in the embryos did not differ either. This may be because only free BPA was measured, and not total BPA (since some BPA is conjugated) and could partially explain the difference between the BPA concentration administered to the females and that which was measured in the embryos. Additionally, the small liver tissue samples from each embryo (which could induces errors in estimating the BPA content) could also contribute to this discrepancy. Nevertheless, primary goal of this experimentation was, indeed, to demonstrate that BPA is detectable not only in the treated mothers, but also in their offspring. Further experimentation is ongoing in order to better define this phenomenon.

In conclusion, we describe for the first time an endometriosis-like phenotype in mice, strongly enhanced by pre-natal exposition to the endocrine disruptor BPA. To the best of our knowledge, this is the first animal model of endometriosis in mice. The data presented, together with our previous observation of the existence of fetal endometriosis in humans (Signorile et al., 2009b, 2010), may induce to reconsider the pathogenesis of endometriosis and to dramatically change the approach to the disease. Considering the high incidence of endometriosis in humans and the enormous problems caused by associated infertility, these observations should be discussed and possibly confirmed in greater experimental settings and the molecular basis carefully dissected.

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