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Exposure to environmental concentrations of hexachlorobenzene induces alterations associated with endometriosis progression in a rat model

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ABSTRACT

Hexachlorobenzene (HCB) is a dioxin-like compound widely distributed and is a weak ligand of the aryl hydrocarbon receptor (AhR). Endometriosis is a disease characterized by growth of endometrial tissue in ectopic sites. Our aim was to investigate the impact of HCB on the endocrine, invasion and inflammatory parameters in a rat endometriosis model surgically induced. Female rats were exposed to HCB (1, 10 and 100 mg/kg b.w.) during 30 days. Results showed that HCB increases endometriotic like-lesions (L) volume in a dose-dependent manner. In L, HCB10 increases microvessel density (immunohistochemistry) and the vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2) and AhR levels (Western Blot), while HCB1 enhances aromatase expression (Western Blot). In addition, in eutopic endometrium (EU), HCB10/HCB100 augments microvessel density, VEGF and MMP-9 expression, while HCB1/HCB10 increases tumor necrosis factor-α (TNF-α) content in peritoneal fluid (ELISA). Interestingly, both L and EU from HCB-treated rats exhibited higher estrogen receptor α (ERα) (immunohistochemistry) and metalloproteases (MMP)-2 and −9 levels (Western Blot), as well as lower progesterone receptor (PR) expression (immunohistochemistry) than in control rats. Environmentally relevant concentrations of HCB could contribute to abnormal changes associated with endometriosis progression and development.

1. Introduction

Endometriosis is a chronic condition associated with debilitating pain and subfertility, defined by the presence of endometrial-like tissue (lesions) outside the uterus (Horne et al., 2017). Despite intensive research, the pathogenesis of endometriosis remains unclear. Growing evidence suggests that endocrine disrupting chemicals (EDCs) may be etiologically involved in the development and severity of disease (Smarr et al., 2016). EDCs may mimic, block or modulate the synthesis, release, transport, metabolism and binding or elimination of natural hormones. Hexachlorobenzene (HCB) is a pollutant which was used as a fungicide and is still released into the environment as a byproduct of several industrial processes (ATSDR, 2015). HCB has been detected in mother's milk (Der Parsehian, 2008) and in serum from post-partum mothers (Bravo et al., 2017). Animal exposure to HCB induces thyroid disruption (Chiappini et al., 2009) and cell invasion and metastasis in breast cancer models (Pontillo et al., 2013). HCB is a dioxin-like compound and a weak ligand of the aryl hydrocarbon receptor (AhR) (Hahn et al., 1989), a transcription factor which triggers membrane and nuclear functions modulating processes such as inflammation, proliferation and migration (Dietrich et al., 2010). It has been suggested that AhR is involved in the normal function of the endometrium, possibly by modulating cellular proliferation in response to hormones (Hernández-Ochoa et al., 2009).

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Endometriosis development and maintenance is dependent on the recruitment of blood vessels to the endometriotic lesions through angiogenesis. Angiogenic vascular endothelial growth factor (VEGF) is increased in the peritoneal fluid of women with endometriosis as compared to healthy women (Pupo-Nogueira et al., 2007). Cyclooxygenase (COX) is the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins (PGs). COX-2 is induced in response to inflammatory stimuli, and its overexpression in endometriosis has been previously documented (Chishima et al., 2002). Matrix metalloproteinases (MMPs) are essential in the physiological function of the endometrium, and endometriosis severity is primarily associated with the degree of expression or activity of MMP-2 and MMP-9 (Salata et al., 2008). The inhibition of COX-2 decreases migration and invasion of endometriotic epithelial and stromal cells through the suppression of MMP-2 and MMP-9 activity, related to the decrease of prostaglandin E_2 (PGE_2) levels (Banu et al., 2008). In addition, the PGE_2 receptors EP2/EP4 pathway promotes phosphorylation of c-Src kinase, triggering MMP activation (Lee et al., 2011). Tumor necrosis factor-α (TNF-α) is a major pro-inflammatory cytokine also implicated in endometriosis, as it induces endometrial cell proliferation and adhesion to peritoneal cells, regulates MMPs and stimulates angiogenesis (Herington et al., 2011).

Aromatase is the key enzyme in the biosynthesis of estrogen, the essential hormone for the establishment and growth of endometriosis (Delvoux et al., 2009). In endometriosis cells, aromatase expression is induced via the COX-2-PGE_2 pathway. Furthermore, estradiol stimulates COX-2 expression by generating a positive feedback loop (Tamura et al., 2004). Aberrant expression and signaling of estrogen and progesterone receptors (ER, PR) have been associated with the progression of endometriosis, which is commonly referred to as estrogen-dependent and progesterone-resistant (Bulun et al., 2015). In previous studies, we have found that HCB increases MMP-2 and MMP-9 activities in human endometrial stromal cell line T-HESC and primary cultures of endometrial stromal cells from eutopic endometrium of control women and patients with endometriosis. Moreover, HCB exposure increases COX-2 and EP4 expression, PGE_2 secretion and c-Src kinase activation in T-HESC, via the AhR pathway (Chiappini et al., 2016). Given that rat endometriotic tissues are similar to human lesions in vivo, the rat model allows to study mechanisms in a controlled manner free from confounding influences such as individual patient variation (Machado et al., 2010).

In this context, our aim was to examine HCB action on endocrine, inflammatory and invasion parameters that contribute to the progression or worsening of endometriosis in an experimental model in rats. To such end, we studied eutopic endometrium (EU) from uterus samples and endometriotic like-lesions (L), evaluating microvessel density, MMP-2 and MMP-9 expression, VEGF, COX-2, AhR and aromatase protein levels. In addition, we analyzed ERα and PR expression and localization, as well as TNF-α content in peritoneal fluid.

2. Materials and methods

2.1. Animals

Virgin female Sprague Dawley rats of 30 days of age were purchased from Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires. Rats were fed Diet 3 rat chow (Asociación Cooperativas Argentinas, División Nutrición Animal) and water ad libitum, and were maintained in a controlled environment of 24 ± 2 °C, 50 ± 10% relative humidity and a 12-12 h light/dark cycle. Animals were acclimatized to these conditions for one week. Three independent experiments were carried out with 6–8 animals per group. All the experiments involving animals were performed following the guidelines established by the Committee of Animal Care and Use at Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina (CICUAL Nro 58730/2013).

2.2. Induction of experimental endometriosis and pesticide exposure

Endometriosis was surgically induced by transplanting autologous fragments of uterine tissue onto the surface of the bowel mesothelium (Vernon and Wilson, 1985). All procedures were carried out under sterile surgical conditions. The uterine horns were identified bilaterally, one of them was attached on the gut and the other one was kept to evaluate the effects of pesticide exposure at the end of the experiment. Three uterine fragments (5 × 5 mm squares with myometrium preserved) were sutured to the surface of the bowel mesothelium (Vernon and Wilson, 1985). Rats were maintained during 15 days in recovery. After surgery, seven-week-old virgin female rats were randomly separated in four groups (control, HCB1, HCB10 and HCB100 mg/kg body weight, b.w.) with 6–8 animals per group. HCB (> 99% purity, from Aldrich-Chemie GmbH & Co, Steinheim, Germany) was administered as a suspension in water containing Tween 20 (0.5/100 ml), by gavage three times a week during 30 days. Control animals received equal volumes of the solvent (Tween 20) by the same route. HCB treatment had no effect on the general health of the animals, as shown by rat's healthy appearance and normal body weight and water and food consumption. HCB (100 mg/kg b.w.) induces preneoplastic lesions in mammary gland and increased mammary tumor growth in rats (Peña et al., 2012). In addition, the HCB doses (1 and 10 mg/kg b.w.) used in the current study are similar to those found in human serum samples from different populations (Guo et al., 2014). Only 6% HCB is absorbed when administered in aqueous suspension (ATSDR, 2015). Although in this study, the HCB internal dose in rats was not measured, we think that this should be similar those found in human populations. This is because in our previous study, when HCB (500 mg/kg b.w. in water solution) was administered to rats by gavage, the serum HCB concentration was 600 ng/ml (Chiappini et al., 2009). In this respect, the HCB concentration observed in human serum samples from the general population in France was 150 ng/ml (Saoudi et al., 2014), therefore it is reasonable to assume that the HCB doses utilized in the present study are environmentally relevant to the general population.

2.3. Evaluation of ectopic uterine tissue

After treatment, stages of the estrous cycle were determined using vaginal smears and the animals were euthanized on the morning of the first estrous stage in CO_2 chamber. All viable L were identified and photographed with a digital camera. The L were measured with a caliper in two dimensions (L: length and W: width, in millimeters), and the spherical volume of each implant was calculated using the formula ($\pi LW2/6$) (Pontillo et al., 2013). The number of viable L for each animal was recorded. The L and uterine horns were excised; one piece was separated for histological studies and immediately fixed in 10% buffered formalin for 24 h, while the other piece was kept in −20 °C for Western blot studies.

2.4. Histology and immunohistochemistry

For immunohistochemical analysis, sections of L and EU were deparaffinized in xylene and rehydrated through graded alcohols, followed by microwaving in 10 mM sodium citrate buffer for antigen retrieval. Non specific staining was blocked by incubation of the sections with Super Block (UltraTek HRP Anti-Polyvalent Lab Pack, ScyTek Laboratories) for 10 min at room temperature (Miret et al., 2017). Tissue sections were incubated with Von Willebrand Factor (1:50, Dako Laboratories), ERα (1:50, Chemicon International Inc.) and PR (1:50, Santa Cruz Biotechnology) antibodies overnight at 4 °C. Three slides for each animal were analyzed and all tissue sections were examined by two blinded observers using a × 600, × 400 and × 100 objective lens on a light microscope (Nikon) connected to a digital camera. Ten fields of immunostained sections were randomly chosen and captured from each specimen. Quantification of the positive cells/total cells ratio was

normalized to control values (vehicule) and expressed as fold control. At least 2500–3000 cells were counted per treatment.

2.5. Homogenization of tissues

EU and L were homogenized immediately after collection in an Ultra-Turrax homogenizer (IKA) in 20 mM Tris buffer (pH 7.4) containing 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mg/kg benzamidine, 1 mM DTT and 10 μg/ml −1 soybean trypsin inhibitor. Then, the samples were sonicated for 10 s. The homogenate was spun at 7800 g for 10 min at 4 °C to remove debris and the supernatants were collected and kept at −70 °C until Western blot analysis.

2.6. Western blotting

Total proteins were prepared, protein concentration was determined and 50–100 μg protein was resolved by 6–10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories). The membranes were then blotted for COX-2 (1:500, Abcam), VEGF (1:500, Abcam), MMP-2 (1:500, Santa Cruz Biotechnology), MMP-9 (1:500, Millipore), aromatase (1:500, Abcam), AhR (1:500, Abcam), and β-Actin (1:2000, Sigma-Aldrich) as previously described (Pontillo et al., 2013).

2.7. TNF-α quantification by ELISA

Levels of TNF-α in peritoneal fluid was assessed by double antibody sandwich Enzyme linked immunosorbent assay using Quantikine ELISA Kits (R&D Systems) according to the manufacturer's instructions. Standard curves were prepared before the antibody reaction. Peritoneal fluid samples were diluted two-fold with Calibrator Diluent RD6U. The wells were read at 450 nm with a Model 550 Microplate Reader (Bio-Rad). Each reaction was run in triplicate. Data were relativized to control values.

2.8. Statistical analysis

Data were evaluated by one-way ANOVA and Tukey's post-hoc test to identify the effect of treatment. Differences were considered significant when p values were < 0.05. Results represent the mean ± SD of at least three independent experiments.

3. Results

3.1. Endometrial implant volume

Rat endometriosis models have been previously reported to replicate human endometriosis pathophysiology (Machado et al., 2010). In the present study, the endometrial implants were established in 95% of similar manner in all animal groups. Morphological analyses revealed ovoid shaped implants, filled with fluid and well vascularized (see Fig. 1), while histopathological studies showed typical endometrial components such as glands and stroma (see Fig. 4). HCB induced a significant dose-dependent increase in implant volume, i.e. 97% for HCB1, 190% for HCB10 and 295% for HCB100. Likewise, animals treated with HCB presented external lesions with greater vascularization and a reddish color in some cases (Fig. 1A).

3.2. Microvessel density in eutopic and ectopic endometrium

Several authors have described angiogenesis as a key step in endometriosis development (Jana et al., 2016). In this report, numerous peritoneal blood vessels were observed around the active endometriotic lesions. To study whether HCB exposure altered vascular density in L and EU, vascularized area and number of vessels were analyzed by von Willebrand (vW) immunohistochemistry. To evaluate the vascularized area, we analyze photographs of EU and L of each animal, by summing the areas corresponding to the blood vessels on the total area of the tissue, using image J programe. To assess the number of blood vessels, we quantified number of vessels and then relativized it to the total area. Our results show that in L, HCB significantly expanded the vascularized area at all the doses assayed, showing a highest 750% increase at HCB10. Similarly, all three HCB doses increased the number of vessels by 51%, 45% and 98%, respectively (Fig. 1B). In EU, HCB significantly augmented the vascularized area in a dose-dependent manner, with a 140% increase for HCB10 and 235% for HCB100. In addition, the pesticide enhanced the number of vessels by 170% at HCB10 and 206% at HCB100 (Fig. 1B).

3.3. Action of HCB on VEGF, COX-2 and MMPs protein expression levels

Given that HCB incremented microvessel density in L and EU, and taking into account that VEGF is an important mediator of angiogenesis in endometriosis (Pupo-Nogueira et al., 2007), VEGF protein levels were evaluated by Western Blot. HCB1 and HCB10 significantly enhanced VEGF expression in L by 115% and 294%, respectively; while HCB10 and HCB100 augmented VEGF content in EU by 350 and 342%, respectively, with similar levels of VEGF stimulation in both samples (Fig. 2A). As COX-2 overexpression is known to contribute to the pathophysiology of endometriosis (Chishima et al., 2002), changes in COX-2 levels were examined upon pesticide exposure. HCB increases COX-2 expression by 350% with HCB10 and 190% with HCB100 in L, and by 210% with HCB1 in EU, thus inducing levels of stimulation greater in L than in EU (Fig. 2B).

Next, and as alterations in MMP-9 and MMP-2 expression are also key in the development of endometriosis (Chung et al., 2002; Collette et al., 2006), MMP levels were evaluated after HCB treatment. Results showed a 40% increase in MMP-2 expression induced by HCB10 and a 50% enhancement induced by HCB100 in L, as well as a 65% increase triggered by HCB100 in EU (Fig. 2C). Moreover, pesticide exposure enhances MMP-9 protein expression by 25% for HCB10 and 48% for HCB100 in L, and by 45% for HCB10 and 60% for HCB100 in EU (Fig. 2C).

3.4. HCB exposure alters AhR, aromatase and TNF-α levels

Considering that AhR can mediate the toxicity of endocrine disruptors with xenoestrogenic activity (Van der Heiden et al., 2009), and that HCB is a weak ligand of AhR (Hahn et al., 1989), studies next focused on AhR protein levels. HCB exposure significantly augments AhR expression by 200% for HCB10 and 160% for HCB100 in L. In contrast, pesticide exposure reduces AhR levels in EU, with a 75% decrease for HCB10 and a 78% for HCB100 (Fig. 3A). Taking into account that aromatase is overexpressed in both eutopic and ectopic endometrium of patients with endometriosis (Velasco et al., 2006), assays were conducted on pesticide effects on aromatase protein expression, with results showing a 470% upregulation of aromatase levels for HCB1 in L, and no alterations in EU (Fig. 3B).

Elevated levels of TNF-α have been reported in the peritoneal fluid and serum of endometriosis patients, contributing to the neoangiogenesis of endometriotic implants (Herington et al., 2011). In the light of this evidence, TNF-α content was analyzed in peritoneal fluid by ELISA, with results rendering significant HCB-induced increases of 25% and 55% in this cytokine at HCB1 and HCB10, respectively (Fig. 3C).

3.5. ERα and PR content in eutopic and ectopic endometrium

ERα activation may occur via both genomic and non genomic mechanisms, resulting in growth-promoting effects in several tissues. It has been well established that ERα is more highly expressed than ERβ in human endometrium, and that estrogens mediate proliferation mainly as a consequence of ERα activation (Shang, 2006). In addition, animal

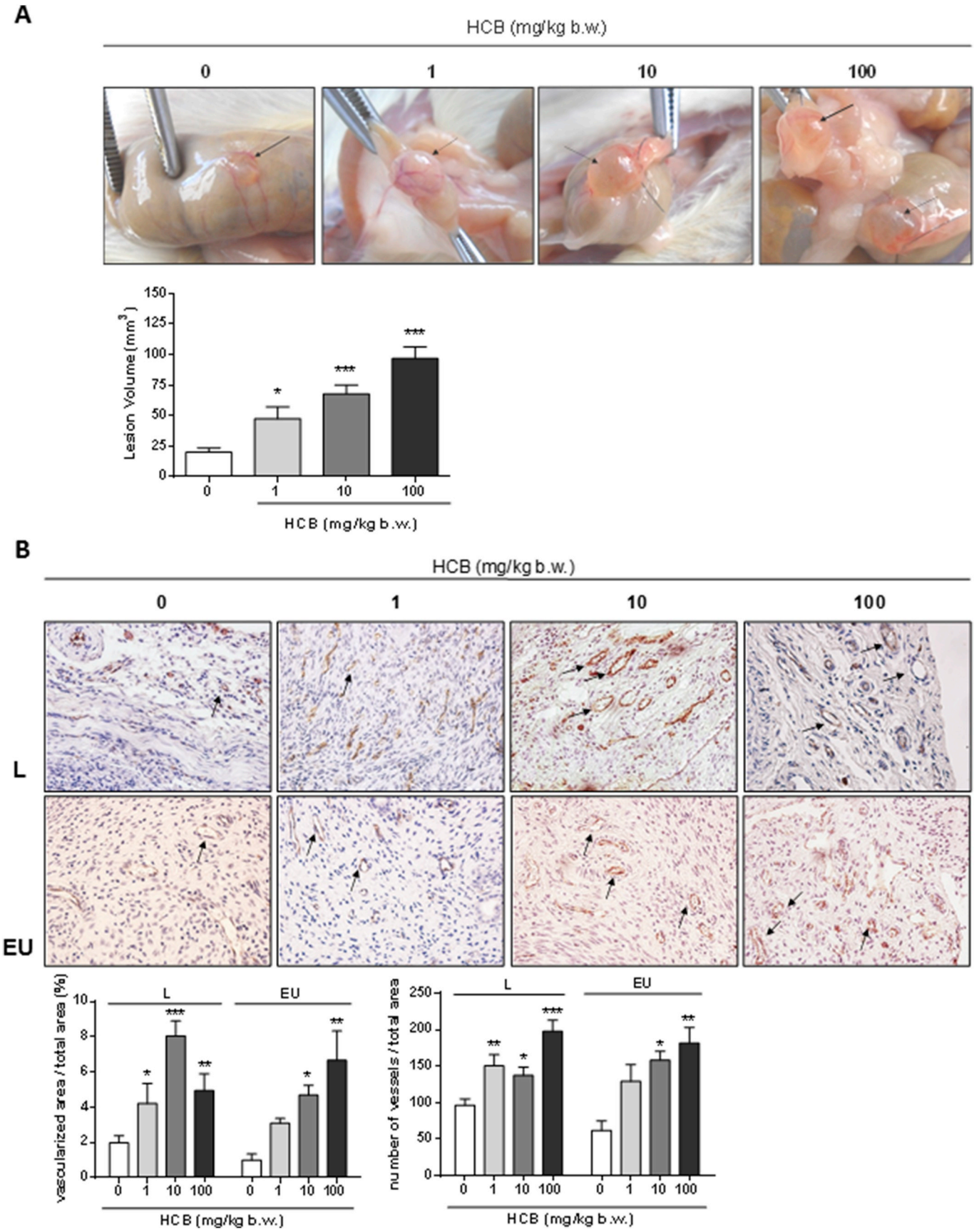

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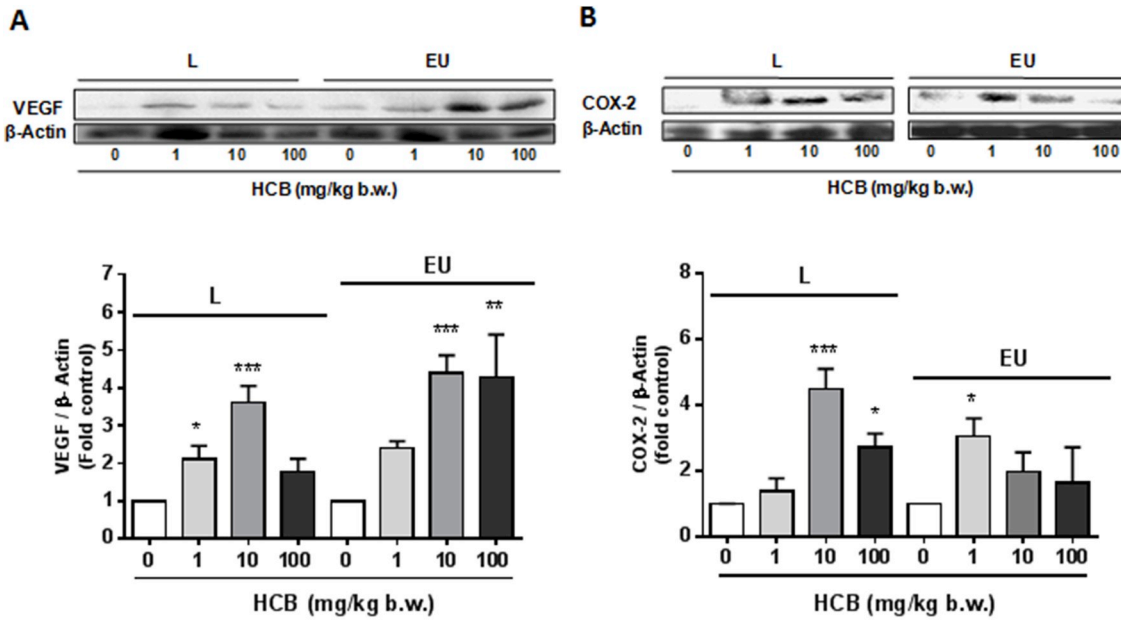
Fig. 1. Volume of L and microvessel density of L and EU in HCB-treated rats. The endometrial fragments were left to grow for 15 days, and then rats were treated with HCB (1, 10 and 100 mg/kg b.w.) or vehicle (control) for 30 days. The animals were euthanized and implants were photographed and measured for analysis. (A) Representative photographs illustrating the characteristics of lesions generated by surgery after HCB treatment are shown in the upper panel. Quantification of lesion volume is shown in the lower panel. The length (L) and width (W) (mm) of each lesion were measured with caliper, and the spherical volume of each implant was calculated using the formula (πLW2/6). (B) Representative images of L and EU microvessels analyzed by vW immunostaining are shown in the upper panels. Sections were incubated with primary antibody ON, rinsed three times and incubated with secondary antibody for 1 h at room temperature. Arrow: vW positive microvessels. Magnification X100. Quantification of vascularized area expressed as vascularized area/total area and number of vessels expressed as number of vessels/total area are shown in the lower panels. Data are expressed as mean ± SD of three independent experiments. Asterisks indicate significant differences vs. respective control (*p < 0.05, **p < 0.01, ***p < 0.001), ANOVA and Tukey's post-hoc test. The color image is available in the online version of the article, and the text is an accurate description of both the black and white and color versions of the figure. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



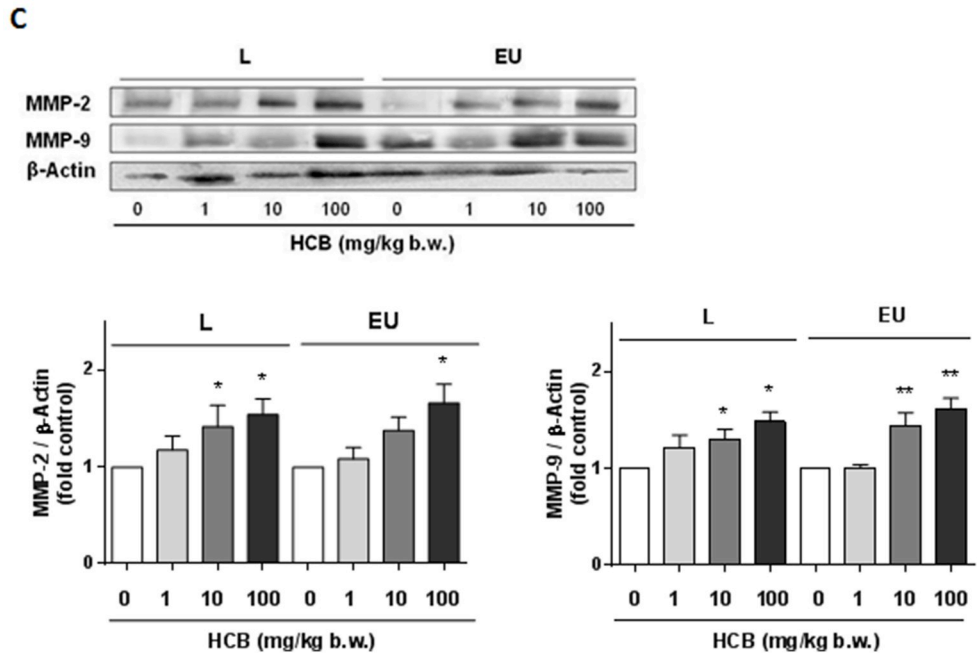


Fig. 2. VEGF, COX-2 and MMP protein expression levels of L and EU in HCB-treated rats. The endometrial fragments were left to grow for 15 days, and then rats were treated with HCB (1, 10 and 100 mg/kg b.w.) or vehicle (control) for 30 days. The animals were euthanized and L and EU were used for protein analysis. (A) VEGF, (B) COX-2, (C) MMP-2 and MMP-9 protein levels. Homogenates of tissues were resolved by SDS-PAGE and immunoblotted for VEGF, COX-2, MMP-2 or MMP-9. Western blot from one representative experiment is shown in the upper panels. Quantification of immunoblots by densitometry scanning is shown in the lower panels. Data are expressed as mean ± SD of at least three independent experiments. Asterisks indicate significant differences vs. respective control (*p < 0.05; **p < 0.01; ***p < 0.001), ANOVA and Tukey's post-hoc test.

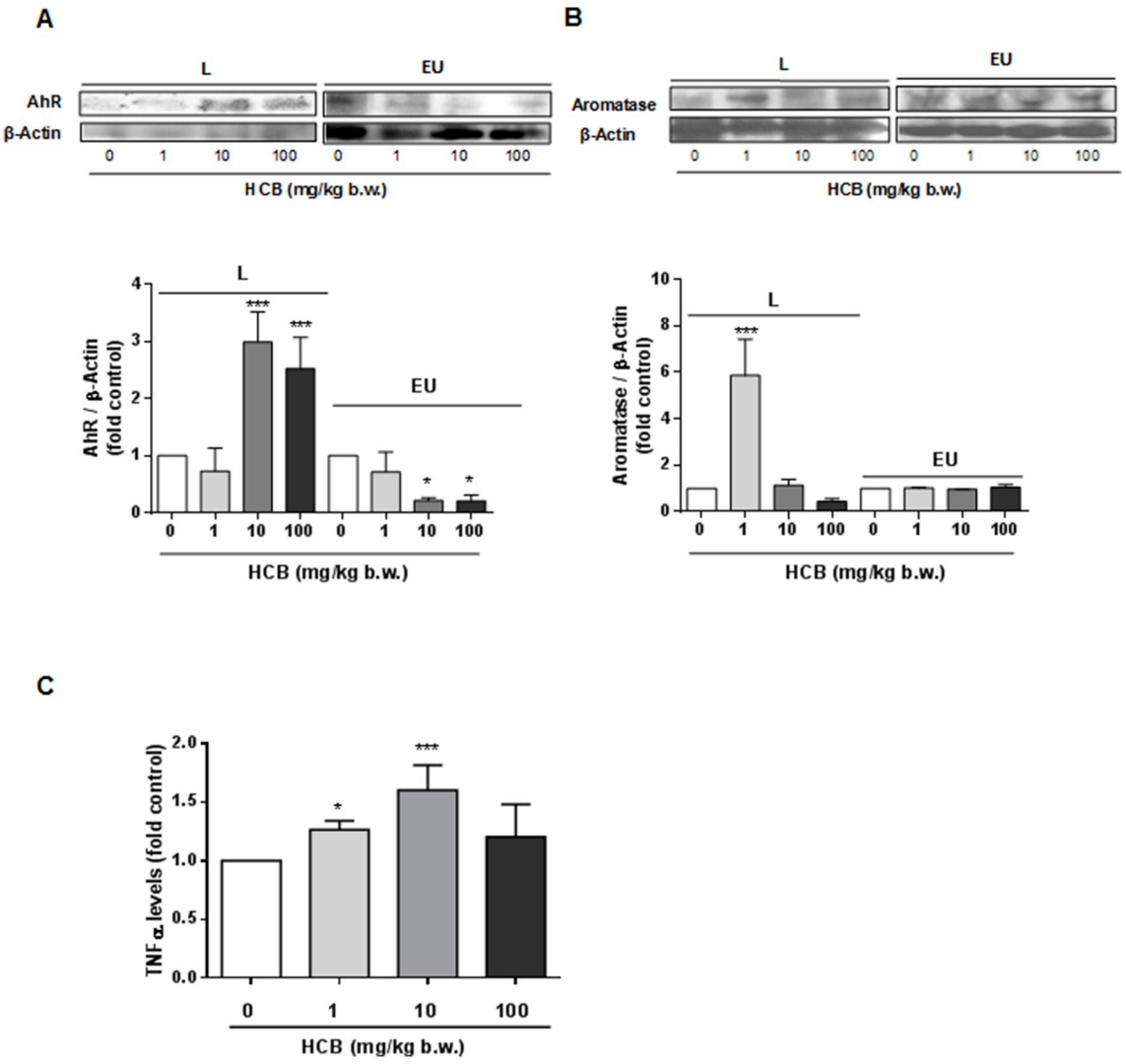


Fig. 3. AhR and aromatase expression of L and EU and TNF-α peritoneal fluid content in HCB-treated rats. The endometrial fragments were left to grow for 15 days, and then rats were treated with HCB (1, 10 and 100 mg/kg b.w.) or vehicle (control) for 30 days. The animals were euthanized and L and EU were used for protein analysis, while the peritoneal fluid was collected. Evaluation of (A) AhR and (B) aromatase protein levels of L and EU by Western blot, and (C) TNF-α content in peritoneal fluid was detected by ELISA. In A and B, homogenates were resolved by SDS-PAGE and immunoblotted for AhR and aromatase. Western blot from one representative experiment is shown in the upper panels. Quantification of immunoblots by densitometry scanning is shown in the lower panels. Data are expressed as means ± SD of at least three independent experiments. Asterisks indicate significant differences vs. respective control (*$p < 0.05$; ***$p < 0.001$), ANOVA and Tukey's post-hoc test.

studies have shown that exposure to environmental toxics may reduce uterine sensitivity to progesterone (Bulun et al., 2006). On the basis of these findings, L and EU slices in the current study were incubated with specific antibodies anti-ERα and PR, and positive cells in stroma (S), glandular epithelium (GE) and luminal epithelium (LE) were then quantified. Immunohistochemistry results revealed higher ERα expression in S (HCB1, 250%; HCB10, 100% and HCB100, 80%) and GE (HCB10, 110% and HCB100, 100%), without changes in LE of L from HCB-treated rats (Fig. 4A). Besides, in samples of EU, HCB induces an enhancement in ERα levels in S (HCB1, 110%; HCB10, 90% and HCB100, 115%), GE (HCB1, 240%; HCB10, 230% and HCB100, 120%) and LE (HCB10, 80% and HCB100, 85%) (Fig. 4B).

On the other hand, HCB exposure reduced the number of PR-positive cells in L, with effects found in GE at all doses assayed (HCB1, 50%; HCB10, 40% and HCB100, 70%), and in LE at HCB10 (35%) and HCB100 (45%) (Fig. 4A). In samples of EU, the pesticide decreased PR expression in at HCB100 in S (30%) and LE (50%) (Fig. 4B).

4. Discussion

Human exposure to organochlorine compounds is a public health concern due to their ubiquitous distribution, high environmental persistence and adverse effects on health (Wigle et al., 2008). Although most of these pollutants have been restricted, they are still found in environmental samples, food and human tissues (Perelló et al., 2015). In recent years, some studies have shown a positive correlation between dioxin-like polychlorinated biphenyls (PCBs), aromatic fungicides and/or HCB and risk of endometriosis (Buck Louis et al., 2012; Upson et al., 2013; Ploteau et al., 2017). In contrast, other authors have found no significant associations with HCB and increased risk of endometriosis (Porpora et al., 2009), while Niskar et al. (2009) have observed levels of serum dioxins are similar in women with and without the disease. These evidences strengthen the biological plausibility that organochlorine pesticides could also contributors to risk of endometriosis.

Although there are limitations with any animal model, the variety of experimental endometriosis models that have been developed has enabled investigation into numerous aspects of this disease. Thanks to

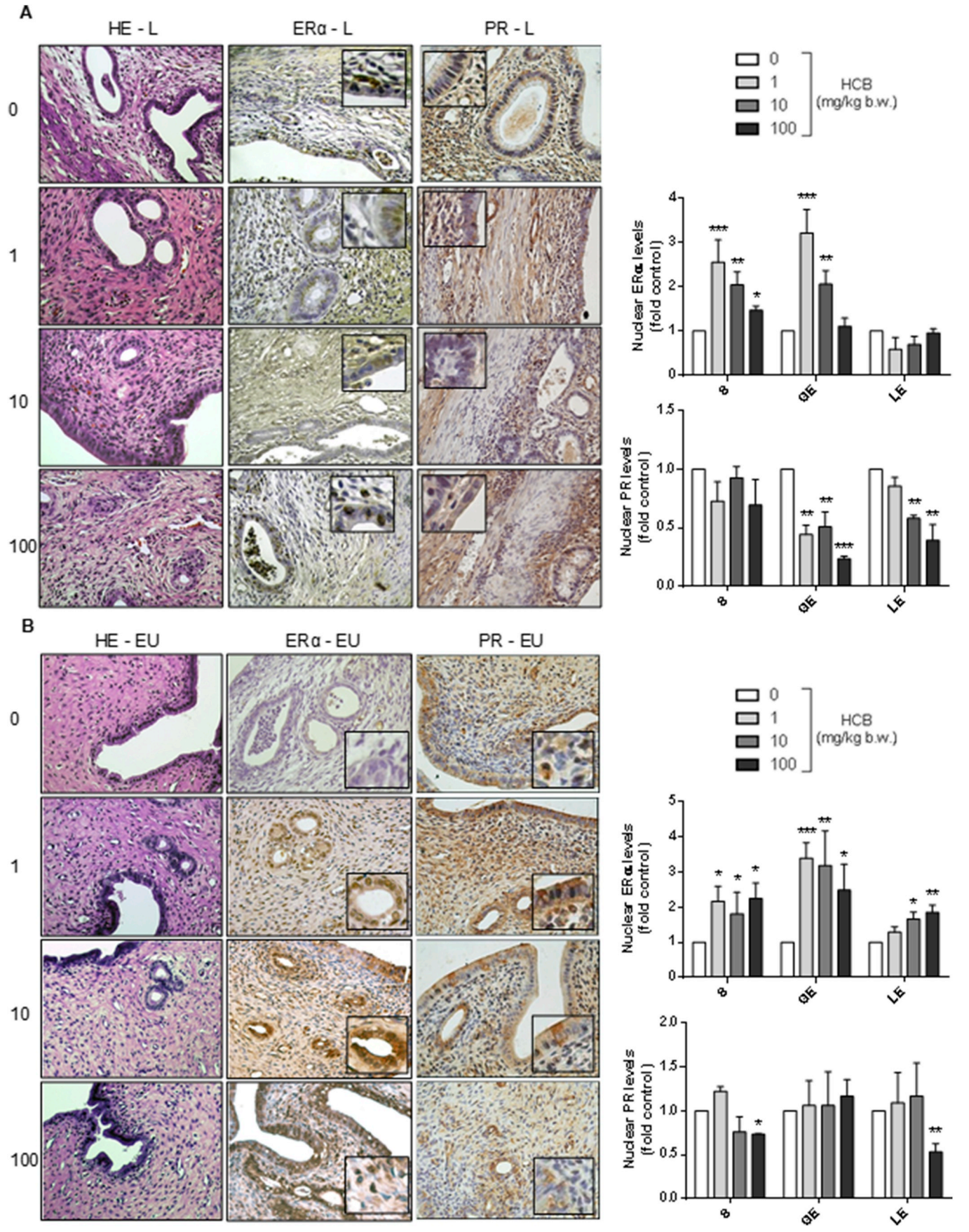


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Fig. 4. Localization and expression of ER-α and PR in HCB-treated rats. The endometrial fragments were left to grow for 15 days, and then rats were treated with HCB (1, 10 and 100 mg/kg b.w.) or vehicle (control) for 30 days. The animals were euthanized, L and EU were fixed and slices were prepared for histological evaluation. Representative images of endometriotic-like lesions (A) and eutopic endometrium (B) sections stained with hematoxylin-eosin, estrogen receptor α (ERα), and progesterone receptor (PR) are shown in the left panels. For immunohistochemical studies, signal detection was carried out using DAB substrate kit and lightly counterstaining with hematoxylin. Quantification of positive cells normalized to control in stroma (S), glandular epithelium (GE) and luminal epithelium (LE) are shown in the right panels. Data are expressed as mean ± SD of three independent experiments with n = 5 rat per group. Asterisks indicate significant differences vs respective control rat (*p < 0.05, **p < 0.01, ***p < 0.001), ANOVA and Tukey's post-hoc test.

animal models, our understanding of the role of steroid responsiveness, inflammatory processes and the peritoneal environment has been advanced (Bruner-Tran et al., 2018). In particular, the current study carried out in a model of rat endometriosis supports the hypothesis that organochlorine pesticides induce adverse effects, as environmentally relevant HCB concentrations (Bravo et al., 2017; Guo et al., 2014) produced alterations in EU and L. We observed that these implants show histological features of human endometriosis, including highly vascularized lesions containing glands and stroma. HCB increases the volume of L in a dose-dependent manner, in line with results showing methoxychlor to promote L growth (Cummings et al., 1995). Other authors have exposed mice to dioxin-type and non-dioxin-type PCBs and found the former to more significantly induce the development of lesions (Huang et al., 2017), suggesting that dioxin-type chemicals triggers endometriosis through AhR mechanism. It has been reported that perinatal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a strong ligand of AhR, increases lesion size in mice (Cummings et al., 1999).

In endometriotic tissue, AhR mRNA levels are higher than in healthy eutopic endometrium (Mariuzzi et al., 2016). The present results show that HCB10 and HCB100 enhance AhR expression, accompanied by increased COX-2 levels in L. This finding is of major interest, as it has been reported that dioxin-like compounds enhance COX-2 mRNA via AhR by binding to xenobiotic response element (XRE) sites on COX-2 promoter (Yoshioka et al., 2011). It appears that events resulting in an increase in AhR levels potentiate the duration and magnitude of the induction of AhR-responsive genes, such as COX-2. In addition, TCDD augments AhR expression and promotes lesions possibly involved in local maintenance of vascularity (Kitajima et al., 2004). Recently, we have reported that HCB enhances AhR levels in human endometrial stromal cell line T-HESC (Chiappini et al., 2016), while our current results show that HCB reduces AhR expression at the same doses in EU, in line with reports showing lower AhR content in liver and lung of TCDD-exposed rats (Pollenz et al., 1998). It is known that AhR down-regulation requires ligand binding both in vivo and in vitro, and that it involves ubiquitination and proteasome (Pollenz, 2002). Also, the inhibition of COX-2 prevents endometriosis and decreases lesions size in different animal models (Matsuzaki et al., 2004). Herein, we observed a significant increase in COX-2 levels in HCB-treated rats, with a greater raise in L than in EU. In agreement with our results, Zidan et al. (2015) have reported higher COX-2 mRNA levels in L compared to EU, while other authors have observed more frequent COX-2 staining in the implants than in EU (Chishima et al., 2002). We have demonstrated that HCB enhances COX-2 levels in endometrial stromal cells from eutopic endometrium of control and patients with endometriosis, as well as in T-HESC, in this case through AhR pathway (Chiappini et al., 2016).

Angiogenesis involves cell proliferation and migration to form new vessels from pre-existing ones. Endometriotic tissue also require these steps to connect endometriotic vessels with the vascular network of the ectopic endometrial sites (Jana et al., 2016). Moreover, aberrant neo-angiogenesis associated with pathological tissue growth has been documented in endometriosis (Jana et al., 2016). For this reason, we sought to examine VEGF levels as a key angiogenic agent and found high VEGF expression upon HCB exposure in both, L and EU. Arlier et al. (2017) have observed that VEGF induces ectopic endometrial angiogenesis, while other reports have shown that bisphenol A increases VEGF expression in rat uterus (Long et al., 2001). In our current study, microvessel density analysis demonstrated that angiogenesis induced by HCB is higher in L than in EU, which is probably related to the rapid growth of implants. The vascularized area showed different patterns in L and EU, increasing at all the doses assayed and peaking at HCB10 in L, but enhancing in a dose-dependent manner in EU. The increase in vascularized area induced by HCB in EU could be associated to a larger number of vessels, while other alterations could be considered to take place in L, such as pathologic angiogenesis. Interestingly, HCB induces a similar pattern of vascularized area and VEGF expression in L and EU. Worth highlighting, in L HCB10 promotes a sharp increase in the expression of VEGF and COX-2, which correlates with the expansion in the vascularized area. These findings are in agreement with reports showing VEGF as a good marker of new blood vessel development in rat endometriosis (Machado et al., 2010).

Accumulating evidence has demonstrated an association between VEGF, COX-2 and MMP upregulation in endometriosis (Ma et al., 2015), with increased levels of MMP-2 and MMP-9 particularly reported at advanced stages of the disease (Salata et al., 2008). Jana et al. (2016) have observed the specific role of MMP-2 in endometriosis-associated angiogenesis. In the current study, we found HCB to increase MMP-2 and MMP-9 expression in L and EU, with similar levels in both samples. Regarding the association between VEGF, COX-2 and MMPs, the present work shows that HCB10 and HCB100 augment VEGF and MMPs expression in EU, with no changes in COX-2. In contrast, HCB10 enhances VEGF, COX-2 and MMP levels in L. Similarly, we have previously observed that HCB upregulates MMP-2/MMP-9 activities and COX-2 expression in T-HESC, HUF cells and endometrial stromal cells from eutopic endometrium of control and patients with endometriosis (Chiappini et al., 2016). Indeed, using human endometrial tissue and an organ culture model, Bruner-Tran et al. (2008) have found that TCDD increases the secretion of MMPs.

TNF-α is elevated in peritoneal fluid of women with endometriosis and has been associated with endometriosis progression and infertility (Darai et al., 2003). Herein, HCB enhances TNF-α content in peritoneal fluid of rats. The highest content of TNF-α was observed in HCB10, coincidentally with an increase in COX-2, VEGF and AhR expression and the vascularized area in L. In this regard, the TNF-α release to the peritoneum could be thought to trigger an inflammatory environment promoting the enhancement in VEGF and COX-2, which might lead to an increase in angiogenesis and consequently in the size of L. In line with our findings, TCDD stimulates secretion of TNF-α in adipocytes through AhR activation (Nishiumi et al., 2010), and TCDD-exposed rhesus monkeys with endometriosis have exhibited higher TNF-α secretion by blood monocytes (Rier et al., 2001). AhR responsive genes also include potent proinflammatory cytokines such as TNF-α (Vogel et al., 2004). Therefore, exposure to AhR ligands could potentially contribute to a chronic pattern of inflammation, possibly resulting in the disruption of normal tissue function.

Aromatase is overexpressed in L and EU of patients with endometriosis as compared to endometrium of healthy women (Velasco et al., 2006). A molecular link exists between inflammation and estrogen production in endometriosis, mediated by a positive feedback cycle which favors expression of aromatase, COX-2 and continuous local production of estrogen and PGE_2 (Tamura et al., 2004). In the current study, HCB augments aromatase levels in L, accompanied by increased COX-2 and ERα expression. Neonicotinoid pesticides have induced aromatase activity in an in vitro model of fetoplacental

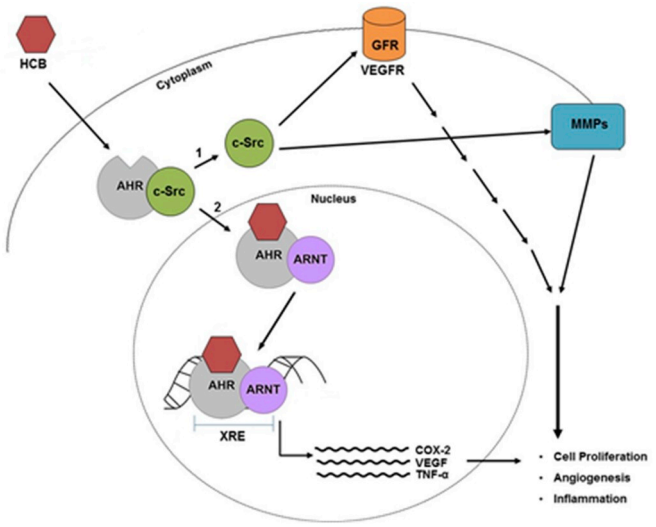

Fig. 5. Mechanism of action of HCB in endometrial stromal and epithelial cells. HCB enters to cells, binds to the AhR-c-Src complex, triggering: 1) a membrane pathway, where c-Src stimulates growth factor receptors (GFR, such as VEGFR); and activates MMPs, promoting cell proliferation and invasion; and 2) a nuclear pathway, where HCB-AhR complex translocates to the nucleus, binds to the AhR nuclear translocator protein (ARNT) and leads COX-2, VEGF and TNFα gene expression (XRE in theirs promoters). This increase in COX-2, VEGF and TNFα expression could induce cell proliferation, inflammation and angiogenesis, contributing to the progression or worsening of endometriosis.

steroidogenesis (Caron-Beaudoin et al., 2017), while other authors have shown that dichloro-diphenyl-dichloroethylene increases aromatase activity in endometrial stromal cells (Holloway et al., 2005). Herein, HCB exerted an estrogenic effect stimulating endometriosis progression, as evidenced by an increased ERα expression in both L and EU. This effect may respond to two distinct mechanisms, either the stimulation of estradiol production through the aromatase expression, or the activation of ERα by AhR coactivation. It has been shown that PR levels are lower in endometriosis compared with eutopic endometrium, generating progesterone resistance (Buck Louis et al., 2012), and the present study revealed reduced PR content in L and EU upon HCB exposure. Other authors have shown that TCDD leads to a progesterone-resistant phenotype in adult mice which can persist for several generations (Bruner-Tran et al., 2010). In addition, the exposure of primary co-cultures of adult human endometrial stromal and epithelial cells to TCDD decreases PR levels in stromal fibroblasts and increases MMP expression in both cell types (Igarashi et al., 2005). Our findings are in accordance with those obtained by these authors, showing that HCB reduces PR content and increases MMP levels in L and EU.

The endocrine disruptors have some properties that have caused controversy, such as low doses may even exert more potent effects than higher doses (Diamanti-Kandarakis et al., 2009). In the present study, we observed that several parameters (VEGF, COX-2, aromatase and ERα expression) were enhanced at lower doses (1 and 10 mg/kg b.w.) than at higher HCB doses. This is interesting, since it would mean that environmental concentrations of HCB are sufficient to induce changes in endometrial cells.

Taking into account our previous studies (Chiappini et al., 2016) and works from others authors (Yoshioka et al., 2011; Roman et al., 2009; Vogel et al., 2004), we propose a mechanism of action in endometrial stromal and epithelial cells. HCB enters to cells, binds to the AhR-c-Src complex, triggering: 1) a membrane pathway, where c-Src stimulates growth factor receptors (GFR, such as VEGFR); and activates MMPs, promoting cell proliferation and invasion; and 2) a nuclear pathway, where HCB-AhR complex translocates to the nucleus, binds to the AhR nuclear translocator protein (ARNT) and leads COX-2, VEGF and TNFα gene expression (XRE in theirs promoters). This increase in COX-2, VEGF and TNFα expression could induce cell proliferation, inflammation and angiogenesis, contributing to the progression or worsening of endometriosis (See Fig. 5).

5. Conclusions

In conclusion, our results show for the first time that environmentally relevant HCB doses alter inflammatory, endocrine and invasion parameters in eutopic endometrium and endometriotic like-lesions in a rat endometriosis model. Environmentally relevant concentrations of HCB could thus have a potential role in the progression of this illness. The HCB would act as a xenoestrogen inducing a proliferative and invasive profile, and a peritoneal proinflammatory and proangiogenic microenvironment contributing to the development and progression of the disease.

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