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Triclosan and bisphenol a affect decidualization of human endometrial stromal cells

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ABSTRACT

In recent years, impaired fertility and endometrium related diseases are increased. Many evidences suggest that environmental pollution might be considered a risk factor for endometrial physiopathology. Among environmental pollutants, endocrine disrupting chemicals (EDCs) act on endocrine system, causing hormonal imbalance which, in turn, leads to female and male reproductive dysfunctions. In this work, we studied the effects of triclosan (TCL) and bisphenol A (BPA), two widespread EDCs, on human endometrial stromal cells (ESCs), derived from endometrial biopsies from woman not affected by endometriosis. Cell proliferation, cell cycle, migration and decidualization mechanisms were investigated. Treatments have been performed with both the EDCs separately or in presence and in absence of progesterone used as decidualization stimulus. Both TCL and BPA did not affect cell proliferation, but they arrested ESCs at G2/M phase of cell cycle enhancing cell migration. TCL and BPA also increased gene expression and protein levels of some decidualization markers, such as insulin growth factor binding protein 1 (IGFBP1) and prolactin (PRL), amplifying the effect of progesterone alone. All together, our data strongly suggest that TCL and BPA might alter human endometrium physiology so affecting fertility and pregnancy outcome.

1. Introduction

Human endometrium undergoes to morphological and functional changes required for a physiological reproduction. Most of these modifications are regulated by the cyclic equilibrium of estrogen and progesterone levels (Martin, 1980; Clarke and Sutherland, 1990). Briefly, when estrogen levels increase endometrial cells begin to proliferate (proliferative phase) (Gellersen and Brosens, 2003; Huhtinen et al., 2012) while when progesterone levels are predominant, stromal endometrial cells differentiate into decidual cells (secretive phase) (Johansson and Wide, 1969; Guerrero et al., 1975; Zhang et al., 1994). The term "decidualization" refers to the specific transformation of the stromal compartment of endometrium necessary to accommodate pregnancy (Gellersen and Brosens, 2014; Paule et al., 2010). Differentiation of stromal cells, indeed, represents the complex initial stage involved in the establishment of a successful pregnancy. So, impaired decidualization causes infertility because the uterus is not able to allow blastocyst implantation and decreases the endometrial receptivity of high quality embryos (Salker et al., 2010, 2012; Gellersen and Brosens, 2014). As a consequence, it is related to implantation and pregnancy failures (Strowitzki et al., 2006; Salker et al., 2011; Weimar et al., 2012).

Age, genetics, diet and environmental pollutants are considered the main risk factors for the onset of endometrial diseases such as endometriosis, endometrial cancer or altered decidualization and abnormal menstrual cycle (Modugno et al., 2005; Vercellini et al., 2014; Signorile et al., 2010). In this regard, many evidences

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suggest that the class of environmental pollutants known as endocrine disruptor chemicals (EDCs) may be responsible for endometrial-linked diseases (Cobellis et al., 2003; Kabbarah et al., 2005; Missmer et al., 2004). EDCs act both on reproductive and non-reproductive systems by mimicking the endogenous hormones essential for the correct physiology of the organism (Diamanti-Kandarakis et al., 2009). Bisphenol A (BPA) and Triclosan (TCL) are well known EDCs, widely used in plastic additives, food industry and personal care products (Rochester, 2013; Rodricks et al., 2010). They accumulate as contaminants in rivers, lakes, seas, groundwater and soil and have been found also in drinking water and food (Fang et al., 2010; Kang et al., 2006; Errico et al., 2014). Furthermore, EDCs concentration in human tissues results increased due to their bioaccumulation through the food chain (Muncke, 2009; Dann and Hontela, 2011). In fact, elevated concentrations of BPA or TCL have been reported in human breast milk, amniotic liquid, blood and urine (Calafat et al., 2008; Dayan, 2007; Henry and Fair, 2013; Nicolucci et al., 2013). Epidemiological studies demonstrated a relation between plasmatic and urine levels of EDCs and the onset of menarche, endometriosis, miscarriages and endometrial cancer (Akesson et al., 2008; Hiroi et al., 2004; Sugiura-Ogasawara et al., 2005). In rats, pre-natal exposure to BPA caused endometriosis-like phenotype in F1 generation (Signorile et al., 2010). Moreover, BPA has been found to affect in mice the placenta patho-physiology and the age of puberty and to stimulate proliferation of endometrial cancer cells (Gertz et al., 2012; Lee et al., 2005; Yang et al., 2014). Clinical studies reported that BPA is associated with miscarriages, decreased blastocyst formation and implantation failure (Sugiura-Ogasawara et al., 2005; Ehrlich et al., 2012a; Ehrilch et al., 2012b). Recently, our group demonstrated in human children, that BPA could also affect metabolic pathways, lipogenesis and insulin production (Menale et al., 2015). On the contrary, to date no findings have been reported in literature regarding the relation between TCL and endometrial pathophysiology. However, it has been demonstrated that TCL exerts both estrogen and anti-estrogen activity *in vitro* and it may be considered as a risk factor for estrogen-related cancer (Ahn et al., 2008; Gee et al., 2008). On the basis of these considerations, in this study we analyzed the effects of TCL and BPA on primary human stromal endometrial cells (ESCs), investigating on cellular proliferation, cell migration and decidualization, three mechanisms strictly linked each others (Petrie et al., 2009; Weimar et al., 2013). ESCs represent a useful cellular model for the analysis of placenta formation (Zhu et al., 2014), since they are able to differentiate *in vitro* under decidualization stimuli, such as progestins and cyclic adenosine monophosphate (cAMP) analogs (Telgmann et al., 1997; Hess et al., 2007). Here we investigated the ability of TCL and BPA to alter the physiological program of endometrium. To this aim *in vitro* experiments have been carried out using different concentrations of TCL or BPA on ESCs both undifferentiated or undergone to decidualization.

2. Materials and methods

2.1. Isolation and culture of ESCs

Endometrial tissues were obtained during gynecological surgery from patients during the proliferative phase of menstrual cycle. Patients were selected for age and for absence of other pathologies linked to endometrium (Table 1). Stromal cells were isolated from a total of 8 samples and different experiments were performed as reported in Table 1. Informed consensus was obtained before collecting the samples. Histological examinations revealed that all endometria were healthy. Isolation of endometrial stromal cells was performed as described by Ryan et al. (1994). Briefly, endometrium samples were minced and digested at 37 °C for 1 h with 2 mg/mL collagenase solution (Sigma Aldrich, St Louis, MO), centrifuged and rinsed with PBS. To remove cellular debris and undigested tissue, the digested tissue was filtered through cell strainers 100 μm and 70 μm in pore diameter, and finally with 40 μm cell strainers in pore diameter. The latter retained epithelial cells, while stromal cells passed through. PBS containing stromal cells was centrifuged at 800 g for 5 min, cells were resuspended in M199 red phenol-free medium (Gibco, UK), supplemented with 10% dextran-coated charcoal FBS (Gibco), 10 nM Estradiol (Sigma), 1X Insulin/Transferrin (Sigma) and antibiotics, cultivated in cell culture dishes in an humidified incubator with 5% CO_2 at 37 °C. When 80% confluence was reached, the cells were enzimatically detached with 0.25% trypsin/EDTA (Euroclone, Milan, Italy) and propagate in culture or frozen. Cells between passage 2 and 8 were used for all the experiments. All the experiments were performed in triplicate.

2.2. Immunofluorescence

The purity of ESCs was assessed by immunofluorescence for vimentin (Santa Cruz Biotechnology, Santa Cruz, CA) and cytokeratin (Santa Cruz) antibodies. 2×10^4 cells per well were plated in 24-well plate. After cell attachment, cells were fixed for 10 min with ice-cold methanol, washed in PBS, blocked with 5% goat normal serum (Santa Cruz) and incubated overnight at 4 °C with vimentin or cytokeratin. Then, Alexa fluor 488 (Invitrogen Carlsbad, CA), 1 h at RT, was used for detection in fluorescence. Cell nuclei were stained with 0.5 μg Höechst (Invitrogen). Images were randomly taken with an inverted fluorescence microscope (Leica Microsystem, Germany).

2.3. Chemicals and treatment

Triclosan (TCL) (Cat Number. PHR1338), Bisphenol A (BPA) (Cat Number. 239658), Progesterone (Pr) (Cat Number. P8783) and Mifepristone (Cat Number. M8046) were purchased from Sigma Aldrich and separately dissolved in ethanol. Chemicals were diluted in complete M199 red phenol-free medium (Gibco 11043-023) and were used for undifferentiated ESCs at the following concentration: TCL or BPA at 10^{-5} M, 10^{-8} M, 10^{-11} M.

Treatments were done for 24 h, 48 h, 72 h as indicated in the appropriate sections. Cells treated with vehicle only (ethanol 0.01%) were used as control. The same treatments on ESCs were also performed after a 24 h Pr pretreatment (10^{-6} M) in order to trigger ESCs decidualization.

2.4. In vitro decidualization

Confluent ESCs were starved with M199 phenol red free with 2% FBS for 24 h and treated for ten day with 10^{-6} M Pr. The medium was replaced every two days. We chose to use progesterone to induce decidualization instead of Br-AMPc (bromo-cyclic adenosine monophosphate cAMP) since it is the physiological stimulus that *in vivo* triggers the decidualization cascade. Then, cells were fixed with formaldehyde 3.7% for 10 min and washed with PBS for the images observation.

2.5. Crystal violet

2×10^4 ESCs were plated in 24 multiwell to evaluate the effects of TCL or BPA on cell proliferation. 24 h post-seeding, cells were serum deprived (FBS 1%) for 24 h and then treated for additional 48 h with TCL or BPA (10^{-5} M, 10^{-8} M, 10^{-11} M). Then, cells were fixed with formaldehyde 3.7% for 10 min, washed with PBS and

Table 1
Patients used for the experiments.

Case no.	Age	Cycle phase	Intervention	Cell culture	Crystal violet	FACS analysis	qPCR	ELISA	Migration
1	38	Proliferative	Hysterectomy	X	X	X	X	X	X
2	49	Proliferative	Hysterectomy	X	X				
3	40	Proliferative	Hysterectomy	X	X	X	X	X	X
4	49	Proliferative	Hysterectomy	X			X	X	X
5	44	Proliferative	Hysterectomy	X		X	X		
6	46	Proliferative	Endometrial ablation	X		X	X		
7	49	Proliferative	Operative hysteroscopy	X	X				X
8	35	Proliferative	Multiple myomectomy	X	X	X	X	X	X

stained with 0.1% crystal violet (Sigma Aldrich) for 10 min. After washing with PBS, acetic acid 10% was used for dissolve crystal violet and optical densities (OD) values at 595 nm were read with a microplate reader.

2.6. FACS analysis

ESCs distribution in cell cycle phases was analyzed by flow citometry. 24 h after serum starvation (FBS 1%), cells were treated for 48 h with TCL or BPA (10^{-5} M, 10^{-8} M, 10^{-11} M). Then, cells were washed with PBS, centrifuged for 5 min at 400 rpm and fixed with 70% Ethanol. Pellet was resuspended in PBS and incubated for 30 min at 37 °C with 100 μg/mL RNAse (Sigma Aldrich) followed by 20 μg/mL propidium iodide (Sigma Aldrich) incubation for 30 min at 4 °C in the dark. Flow citometry was performed using a FACScan™ flow cytometry system (Becton Dickinson, San Jose, CA). For each sample, 5×10^4 events were registered and cell percentage in each phase of cell cycle was calculated.

2.7. RNA extraction and qPCR

Using Trizol, (Life Technologies) RNA from ESCs either treated for 24, 48 and 72 h with TCL or BPA alone (10^{-5} M, 10^{-8} M, 10^{-11} M) or pre-treated for 24 h with Pr (Pr + TCL, Pr + BPA) was extracted. Before quantification DNA residues were depleted using DNAseI (Life Technologies). RNA quality was assessed using Experion system (BioRad). For qPCR assays, from each sample 500 ng of total RNA were retrotranscribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was performed by means of a 7900 HT Real Time PCR (Life Technologies). Primers (Table 2) of genes investigated (*IGFBP1, LEFTY, IL-6, PRL, MMP2, MMP3, MPP11, TIMP2, MMP14, MMP9, TIMP1, TIMP3*) were designed using Primer express 3.0 (Applied Biosystems) and the sequences in GenBank (http://www.ncbi.nlm.nih.gov/genbank). The amount of cDNA target was calculated using the cycle threshold (Ct) and expressed as mean of the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using GAPDH as housekeeping gene.

2.8. Migration assay

Cells were treated for 48 h with TCL or BPA (10^{-8} M) alone or after a 24 h pre-treatment with Pr (10^{-6} M). Then ESCs were trypsinized and allowed to attach in the upper sides of transwell. Cells migration assays were performed using 8 μm pores transwell inserts (BD, Falcon) for a time (24 h) shorter than doubling time of ESCs. Non-migrated cells on the upper side of the transwell were removed by a cotton swab. Migrated cells, attached to the inner side of the transwell, were fixed with formaldehyde 3.7% and stained with 0.1% crystal violet. Cells were counted by two observers in a double blind method (V.M., M.F.) using Image J plugin cell counter (http://imagej.nih.gov/ij/). For each transwell, 5 images (10× enlargement) were taken.

Table 2
Gene amplified by qPCR and sequences of the primer used for their amplification.

Gene		Primer sequence
IGFBP1	Forward	CTATGATGGCTCGAAGGCTC
	Reverse	TTCTTGTTGCAGTTTGGCAG
LEFTY	Forward	AGCCAGAGCTTCCGAGAG
	Reverse	CTCCATGCCGAACACCAG
IL-6	Forward	GCTGCAGGCACAGAACCA
	Reverse	GCTGCGCAGAATGAGATGAG
PRL	Forward	TCTCGCCTTTCTGCTTATTATAACC
	Reverse	GATTCGGCACTTCAGGAGCTT
MMP2	Forward	TTGATGGCATCGCTCAGATC
	Reverse	TGTCACGTGGCGTCACAGT
MMP3	Forward	GAGGCATCCACACCCTAGGTT
	Reverse	ATCAGAAATGGCTGCATCGAT
MPP11	Forward	CTTCCGAGGCAGGGACTACT
	Reverse	AGAAGTCAGGACCCACGAGA
TIMP2	Forward	CCAAGCAGGAGTTTCTCGAC
	Reverse	GACCCATGGGATGAGTGTTT
MMP14	Forward	TCAAGGAGCGCTGGTTCTG
	Reverse	AGGGACGCCTCATCAAACAC
MMP9	Forward	TGGGGGGCAACTCGGC
	Reverse	GGAATGATCTAAGCCCAG
TIMP1	Forward	CTGTTGTTGCTGTGGCTGATA
	Reverse	CCGTCCACAAGCAATGAGT
TIMP3	Forward	ATGGTGTAGACCAGCGTGC
	Reverse	AGGACGCCTTCTGCAACTC
GAPDH	Forward	CAAGGCTGTGGGCAAGGT
	Reverse	GGAAGGCCATGCCAGTGA

2.9. ELISA

To assess IGFBP1 and PRL secretion, ELISA assays were performed using specific kits (Abcam, Cambridge, UK) and according to the manufacturer's instructions. Briefly, 1×10^6 cells were seeded in 100 mm culture dish and treated with BPA or TCL (10^{-8} M) for 48 h alone or pre-treated for 24 h with Pr (10^{-6} M). IGFBP1 and PRL levels were also evaluated in differentiated cells, at the end of the experiment (10th day of treatment) and in ESCs treated with Pr 10^{-6} M, TCL or BPA 10^{-8} in combination with the Pr receptor modulator Mifepristone (10^{-6} M) (Supplementary data). Then, cell culture supernatants were centrifuged for debris removal for the assay. Readings were performed at 450 nm and the values were converted in protein concentration, after generating a calibration curve using appropriate standards.

2.10. Statistical analysis

Statistical analysis was performed using Graph Pad Prism 5.0 (GraphPad Software, San Diego, CA, USA). For each analysis means ± SE of three independent experiments are reported. Significance of multiple treatments was evaluated using the One Way ANOVA with Bonferroni *post hoc*, and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. ESCs proliferation and cell cycle phases

In order to evaluate the ESCs purity, we first analyzed by immunofluorescence the presence of vimentin, a specific marker for endometrial stromal cells (Fig. S1). *In vitro* decidualization was induced treating ESCs cells with Pr (10^{-6} M) and it was completed in 10 days (Fig. S2).

Crystal violet assay was performed to analyze the effects of EDCs on endometrial stromal cells. ESCs treated for 48 h with different concentrations of TCL or BPA (10^{-5} M, 10^{-8} M, 10^{-11} M) did not show significant changes in proliferation (Fig. 1A and B) compared to the control (vehicle 0.01%).

To analyze the cell cycle perturbation induced by EDCs, ESCs exposed for 48 h to TCL or BPA were subjected to cytofluorimetry analysis. Fig. 2 shows representative pictures of cell cycle distribution under the different experimental conditions. 10^{-5} M TCL treatment did not perturb cell cycle phase distribution (Fig. 2A), while decreasing TCL amount (10^{-8} M and 10^{-11} M) significantly induced the switch in G2/M phase of cell cycle (Fig. 2B and C), whit a concomitant decrease of G0/G1 cell fraction. Similarly, BPA treatments (10^{-8} M and 10^{-11} M) increased G2/M and decreased G0/G1 fraction (Fig. 2E and F). No variations were observed after the treatment with BPA at 10^{-5} M (Fig. 2D).

Histograms (Fig. 2H and I) summarize the percentage distributions of the cells in each cycle phase for each experimental condition.

3.2. Gene analysis of decidualization markers

To understand if EDCs could affect the transformation of stromal endometrium we assessed if TCL and BPA were able to deregulate genes involved in decidualization such as *IGFBP1*, *LEFTY*, *PRL*, *IL6*. To this aim the expression of these genes was analyzed by means of qPCR after 24 h (**Panel A**), 48 h (**Panel B**) or 72 h (**Panel C**) of TCL or BPA exposure. Furthermore the effect of both EDCs on gene expression was also analyzed in ESCs pre-treated for 24 h with 10^{-6} M Pr before adding TCL or BPA (Pr + TCL; Pr + BPA), and in the corresponding ESCs control treated with Pr alone. Fig. 3 refers to the expression of *IGFBP1*, Fig. 4 to the expression of *LEFTY*, Fig. 5 to the expression of *PRL* and Fig. S3 to the expression of *IL6*. We did not observe any variation after 24 h of exposure in *IGFBP1* mRNA levels (Fig. 3A). In contrast, TCL increased *IGFBP1* expression after 48 h at all the concentrations used but only at 10^{-8} M and 10^{-11} M in ESCs pre-treated with Pr (Fig. 3B). After 72 h TCL significantly affected *IGFBP1* gene expression at 10^{-5} M and 10^{-8} M in both ESCs treated with TCL alone or pre-treated with Pr (Fig. 3C). *IGFBP1* resulted similarly modulated by BPA 10^{-8} M and 10^{-11} M and by BPA+ Pr at 10^{-8} M after 48 and 72 h of exposure (Fig. 3B and C). Time course analysis for *LEFTY* revealed no change in gene levels after 24 h of treatment (Fig. 4A). 10^{-8} M and 10^{-11} M TCL treatment of ESCs revealed an increased expression of *LEFTY* after 48 and 72 h, while in ESCs pre-treated with Pr it was effective at 10^{-8} M (Fig. 4B and C). BPA affected *LEFTY* expression only at 10^{-8} M after 48 h of treatment but no additive effects were detected with Pr (Fig. 4B). *PRL* expression did not change after 24 h of treatment (Fig. 5A). TCL increased *PRL* expression after 48 h of treatment at 10^{-8} M and 10^{-11} M (Fig. 5B). At 72 h the effect persisted only at 10^{-8} M (Fig. 5C). *PRL* mRNA levels resulted increased after BPA 10^{-8} M treatment at 48 h and at 10^{-8} M and 10^{-11} M after 72 h (Fig. 5B and C). In this case, we observed a synergistic effect in ESCs pre-treated with Pr and treated with BPA 10^{-8} M after 72 h of exposure (Fig. 5C).

We did not observe any difference in the expression levels of *IL-6* in ESCs treated cells compared to control (Fig. S3A–C).

3.3. ESCs migration analysis

To better elucidate if BPA or TCL were able to induce ESCs migration, we analyzed this process investigating by qPCR the expression of genes involved in migration process (Fig. 6) and performing a migration assay after 48 h of treatment on ESCs (Fig. 7). Among metalloprotease genes (*MMPs*) analyzed the mRNA levels of *MMP2* (Fig. 6A), *MMP11* (Fig. 6D) and *MMP14* (Fig. 6E) were unaffected by both treatment with TCL or BPA. However *MMP14* resulted positively regulated by Pr (Fig. 6E). *MMP3* mRNA levels increased after TCL treatment with a significant increase directly linked to TCL concentrations (10^{-5} M and 10^{-8} M) (Fig. 6B). Pr alone down-regulated *MMP3* expression (Fig. 6B), while Pr in combination with TCL (10^{-5} M and 10^{-8} M) determined increased expression of *MMP3* compared to Pr treated cells (Fig. 6B). BPA affected differently the expression of *MMP3* since it resulted effective only at 10^{-8} M (Fig. 6B). TCL (10^{-8} M) and BPA (10^{-5} M and 10^{-8} M) both increased the expression of *MMP9* that resulted up-regulated also in cells treated with Pr alone or in combination with TCL (Fig. 6C). Regarding the expression of tissue inhibitor of metalloproteinase (*TIMP*), only TCL slightly decreased *TIMP1* (Fig. 6F) and dramatically down-regulated *TIMP2*, alone or administered after Pr treatment (Fig. 6G). *TIMP3*, instead, resulted down-regulated in ESCs treated with TCL, BPA (10^{-5} M) and Pr (Fig. 6H).

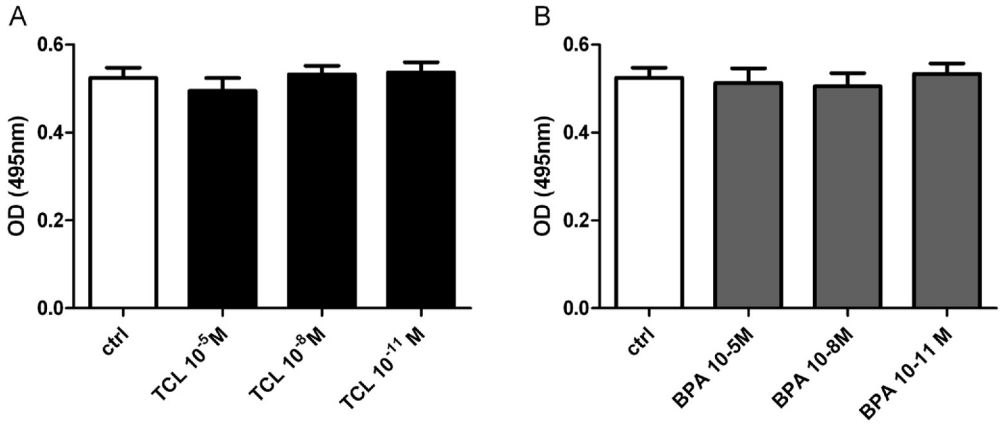

Fig. 1. TCL and BPA effects on ESCs Proliferation. ESCs treated for 48 h with TCL (**A**) and BPA (**B**) were analyzed by crystal violet test. No variations in optical density (OD) were detected in treated cells compared to control.

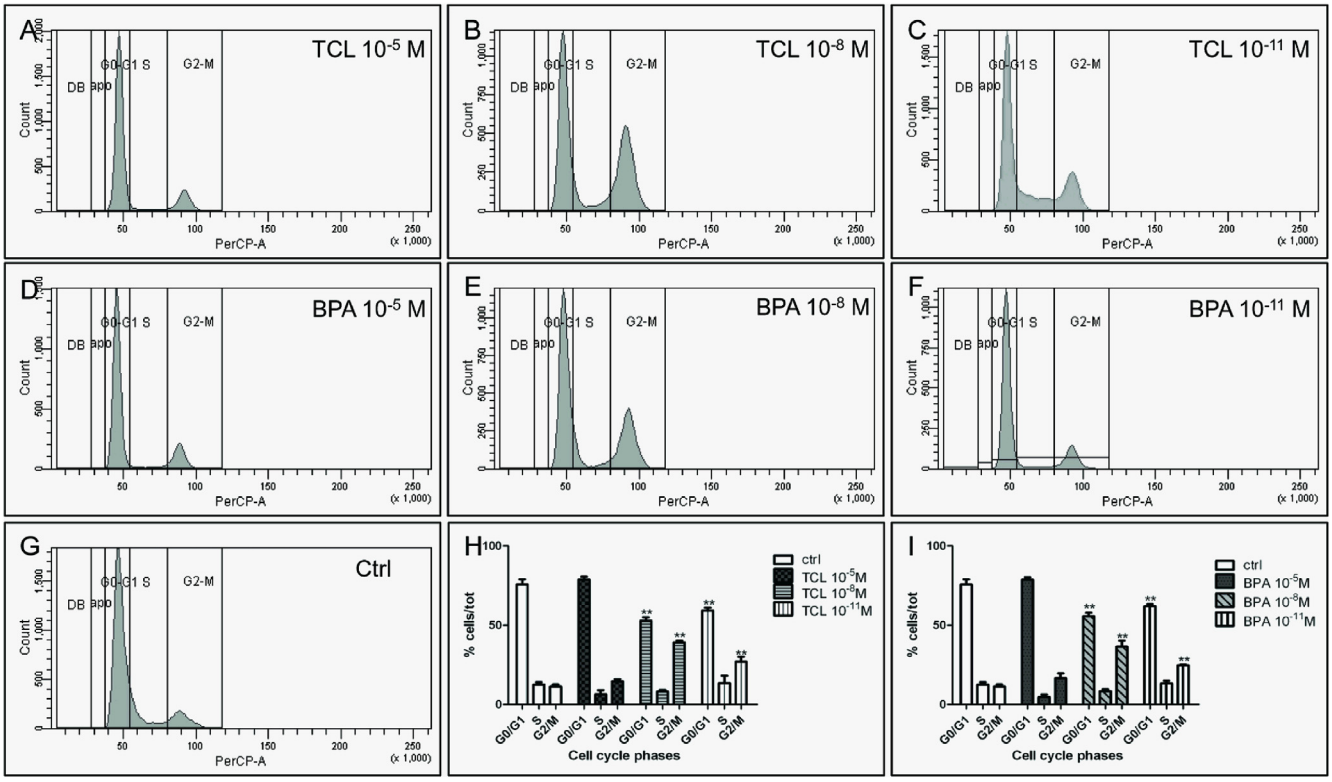

Fig. 2. Cell cycle distribution of ESCs cells after TCL and BPA exposure. Representative histograms (**A**–**G**) and relative data summary (**H, I**) shown an increased percent of cells in G2/M after 10^{-8} M and 10^{-11} M treatment with TCL (**B, C**) and BPA (**E, F**), with a subsequent decreasing in G0/G1 phase. Results were analyzed comparing treated (**A**–**F**) and untreated cells (**G**). Data represent the mean ± SE of three experiments. **P < 0.01 related to control group.

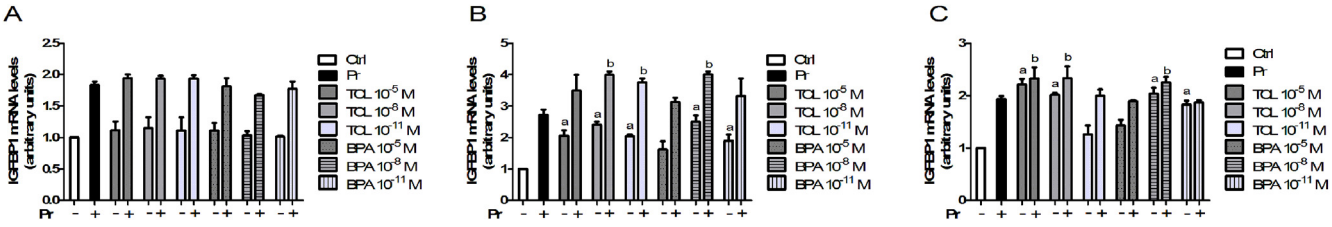

Fig. 3. Gene expression of *IGFBP1* after treatment with TCL or BPA. ESCs cells were treated for 24 h (**Panel A**), 48 h (**Panel B**) and 72 h (**Panel C**) with TCL or BPA alone (10^{-5} M, 10^{-8} M, 10^{-11} M) or after pre-treatment for 24 h with 10^{-6} M Pr (+). Control cells (Ctrl) were treated with vehicle only. *IGFBP1* mRNA levels were normalized using GAPDH as housekeeping gene. a, response significantly different than the vehicle control (P < 0.05); a•, response significantly different than the vehicle control (P < 0.01); b, response significantly different than Pr alone (P < 0.05).

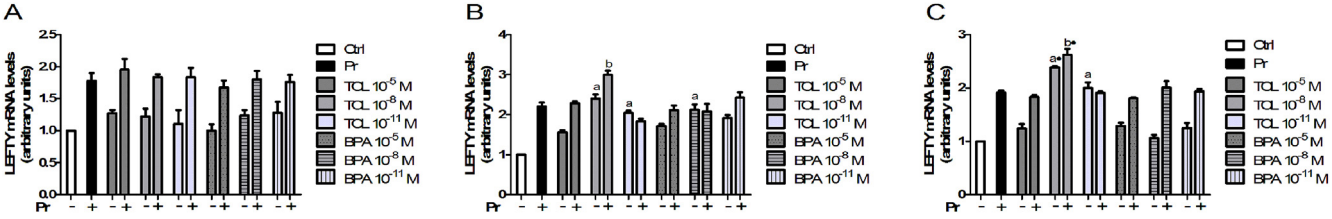

Fig. 4. Gene expression of *LEFTY* after treatment with TCL or BPA. ESCs cells were treated for 24 h (**Panel A**), 48 h (**Panel B**) and 72 h (**Panel C**) with TCL or BPA alone (10^{-5} M, 10^{-8} M, 10^{-11} M) or after pre-treatment for 24 h with 10^{-6} M Pr (+). Control cells (Ctrl) were treated with vehicle only. *LEFTY* mRNA levels were normalized using GAPDH as housekeeping gene. a, response significantly different than the vehicle control (P < 0.05); a•, response significantly different than the vehicle control (P < 0.01); b, response significantly different than Pr alone (P < 0.05).

To verify if gene deregulation of *MMP* and *TIMP* resulted in a modified migratory capability of ESCs, we performed a migration assay treating ESCs at the concentration of 10^{-8} M for TCL or BPA, that showed the highest effect on gene expression. As reported in Fig. 7, TCL or BPA (10^{-8} M) as well as Pr (10^{-6} M) enhanced the migration of ESCs cells, with the high number of migrated cells detected in Pr treated ESCs. Moreover, the highest induction of migration was evident in Pr + TCL. No significant differences were

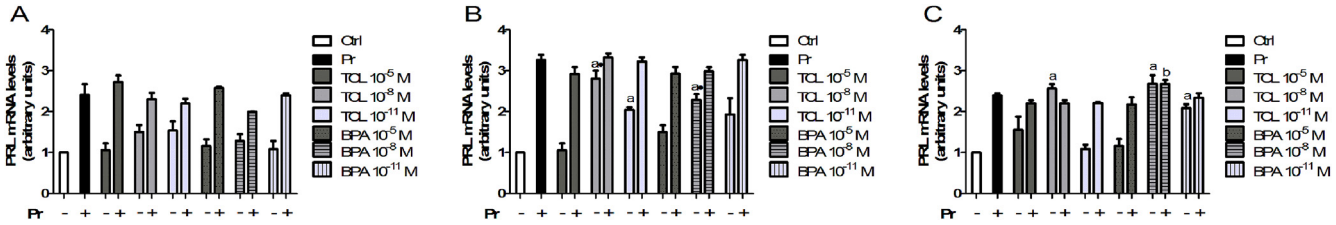

Fig. 5. Gene expression of *PRL* after treatment with TCL or BPA. ESCs cells were treated for 24 h (**Panel A**), 48 h (**Panel B**) and 72 h (**Panel C**) with TCL or BPA alone (10^{-5} M, 10^{-8} M, 10^{-11} M) or after pre-treatment for 24 h with 10^{-6} M Pr (+). Control cells (Ctrl) were treated with vehicle only. *PRL* mRNA levels were normalized using GAPDH as housekeeping gene. a, response significantly different than the vehicle control ($P < 0.05$); a•, response significantly different than the vehicle control ($P < 0.01$); b, response significantly different than Pr alone ($P < 0.05$).

found between Pr + BPA and Pr treated cells.

3.4. IGFBP1 and PRL secretion

To verify if gene deregulation resulted in protein levels variations, we monitored the secretion of IGFBP1 and Prolactin (PRL) after 48 h of treatment using ELISA. As shown in Fig. 8A, Pr, TCL or BPA alone increased the amount of secreted IGFBP1. In combined treatment, Pr + TCL showed the highest level of IGFBP1 compared to Pr + BPA. Also PRL levels (Fig. 8B) resulted notably increased after treatment with Pr. PRL secretion is also enhanced by both TCL and BPA treated cells as well as in Pr + TCL treated cells. Conversely, no additive action was evidenced after the treatment with Pr + BPA, compared to Pr treated cells. In addition, to assess if TCL or BPA affected IGFBP-1 secretion via Pr receptor, we performed an ELISA adding a Pr signaling modulator (Mifepristone 10^{-6} M) in ESCs treated with TCL or BPA. As shown in Fig. S4 Mifepristone reduced the IGFB1 secretion only in Pr treated cells, without inhibiting its secretion in ESCs exposed to TCL or BPA 10^{-8} M.

4. Discussion

Human endometrium is a steroid-responsive tissue subjected to cyclic regeneration involving proliferation, differentiation, breakdown and repair (Norwitz et al., 2001). During each menstrual cycle the endometrium shows a short period of receptivity for successful embryo implantation known as 'implantation window'. This study was aimed to ascertain the effects induced by TCL or BPA exposure on undifferentiated ESCs and ESCs that begin their decidualization process. TCL and BPA belong to the class of EDCs, xenobiotic compounds that interfere with the molecular pathways of living organisms. It is well known that EDCs can increase or decrease the hormone receptor activities (Diamanti-Kandarakis et al., 2009). Several studies in animal models underline the involvement of EDCs on the development of female reproductive system diseases (Mouritsen et al., 2010; Semiz et al., 2008). However, little is known about their effects on human endometrium.

In this work we simulated *in vitro* the physiological cycle of endometrium and evaluated the physiological responses after exposure to relevant environmental and serum doses of TCL or BPA. As reported in literature, human serum BPA concentration ranges between about 10^{-9} M and 10^{-8} M in healthy subjects and in women with recurrent miscarriages, respectively (Takeuchi and Tsutsumi, 2002; Zheng et al., 2012). BPA distribution in other sources has been shown to range between 10^{-11}–10^{-7} M in surface water and effluents and between 10^{-10}–10^{-9} M in drinking water (Michałowicz, 2014). TCL has been demonstrated to be concentrate in human serum at 10^{-8} M (Allmyr et al., 2008), while was found in drinking water at 10^{-10} M (Loraine and Pettigrove, 2006) and in surface water and effluents in a range between 10^{-13}–10^{-8} M (Bedoux et al., 2012). Hence, we decided to perform the experiments using different concentrations of EDCs from 10^{-5} M, usually found in occupational exposure to EDCs (Xiao et al., 2009) to 10^{-11} M. The concentration 10^{-8} M and 10^{-11} M were used to verify the low dose effects (Vandenberg et al., 2007).

We first evaluated the responses of ESCs treated with TCL or BPA alone for 48 h, investigating the proliferation and the cell cycle phases distribution. Several studies reported that BPA increases cell proliferation, but most of these studies analyzed tumorigenic or epithelial cells, such as human endometrial cancer cells and breast cancer cells (Soto et al., 1995; Ricupito et al., 2009; Pisapia et al., 2012); in contrast TCL has been shown to have a dual role in stimulating or inhibiting cell proliferation (Ahn et al., 2008). Our results highlighted non-significant changes in the proliferation profile. On the other hand, TCL and BPA treatments increased cell cycle G2/M phase in ESCs, a clear signal of the beginning of the differentiation process. Data reported in literature have shown that stromal endometrial cells are arrested in G2/M phase after decidualization stimuli, such as progesterone (Logan et al., 2012; Tang et al., 2009). So, we focused our attention on the decidualization process, testing the effect of TCL or BPA on ESCs non completely differentiated but treated for 24 h with Pr prior to start the treatment with EDCs. Decidualization is the mechanism by which stromal cells of endometrium prepare the uterus to accommodate the embryo (Gellersen and Brosens, 2014). Notwithstanding this process in not completely characterized, two observations are already firmly established: i) the morphological changes of stromal cells from fibroblast to epithelioid phenotype (Mannelli et al., 2014) during decidualization; and ii) the increased levels of IGFBP-1, LEFTY and PRL, that are the main markers whose expression increases during decidualization (Gellersen and Brosens, 2014). During this phase, endometrial stromal cells reprogram their cell cycle and acquire a migratory capability needed for the establishment of contact with the trophoblast (Logan et al., 2012).

After treatments, we analyzed the expression of the main markers of decidualization, such as the insulin growth factor binding protein 1 (*IGFBP1*), prolactin (*PRL*), *LEFTY* and the interleukin 6 (*IL-6*). qPCR analysis showed a variable profile of gene expression after the treatment with TCL or BPA. The short time exposure (24 h) did not produce variations in gene levels. In contrast, TCL was found to up-regulate *IGFBP1*, *LEFTY* and *PRL* at 48 and 72 h. Notably, the concentration of TCL that always induced a deregulation of these genes is 10^{-8} M. Moreover, TCL 10^{-8} M is also able to enhance the expression of *IGFB1* and *LEFTY* in ESCs pretreated with Pr. Our experiments demonstrated that BPA also affected gene levels of decidualization markers. Its effect was less significant if compared to TCL. As example, BPA 10^{-8} M enhanced the up-regulation induced by Pr only after 48 h for *IGFBP1* and 72 h for *PRL*. Hence, we decided to verify with ELISA assay if the gene expression of *IGFBP1* and *PRL* resulted in an increased secretion of these two proteins. We performed the assay treating the cells with

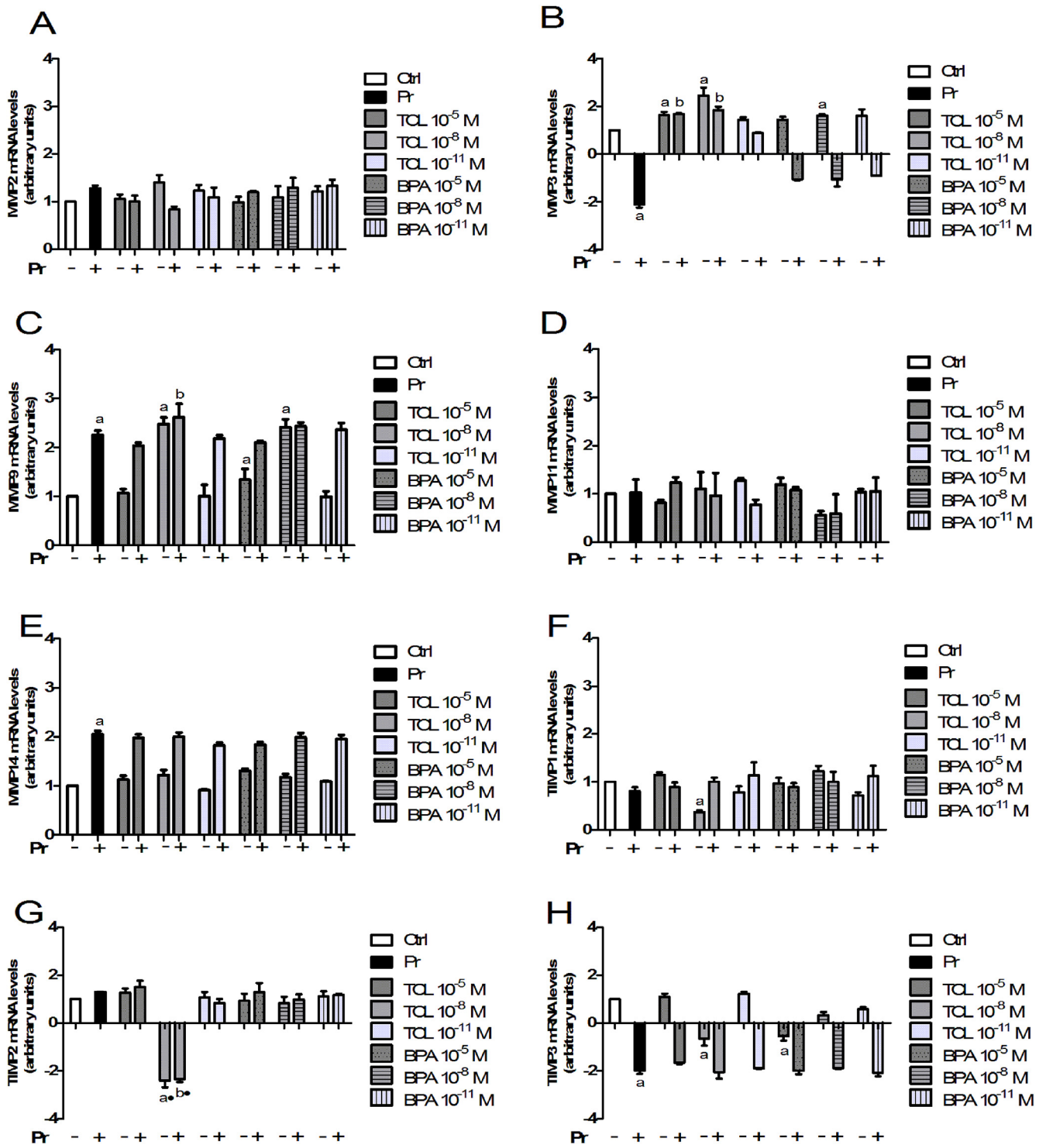

Fig. 6. Gene expression of genes involved in cell migration. ESCs cells were treated for 48 h with TCL and BPA alone (10^{-5} M, 10^{-8} M, 10^{-11} M) or after pre-treatment for 24 h with 10^{-6} M Pr (+). Control cells (Ctrl) were treated with vehicle only. *MMP2* (**A**), *MMP3* (**B**), *MMP9* (**C**), *MMP11* (**D**), *MMP14* (**E**), *TIMP1* (**F**), *TIMP2* (**G**), *TIMP3* (**H**) relative mRNA levels were normalized using GAPDH as housekeeping gene. a, response significantly different than the vehicle control ($P < 0.05$); a•, response significantly different than the vehicle control ($P < 0.01$); b, response significantly different than Pr alone ($P < 0.05$).

concentration of EDCs (10^{-8} M) that showed the most notable effects in gene expression analysis. Both EDCs analyzed increased the secretion of IGFBP1 and PRL. In particular, TCL enhanced the secretion of both IGFBP1 and PRL induced by Pr. Notwithstanding our results did not show a time and a concentration dependent effect, we might speculate that TCL or BPA could mimic the effect of Pr, activating the AMPc-Protein Kinase A pathway. Interestingly, Aghajanova and Giudice (2011) also investigated the effects of BPA on human endometrial stromal cells after 48 h of treatment, although using concentration of BPA much higher (from 10^{-4} M to 5×10^{-6} M) compared to the concentration used in our study. In particular they demonstrated that BPA is able to up-regulated

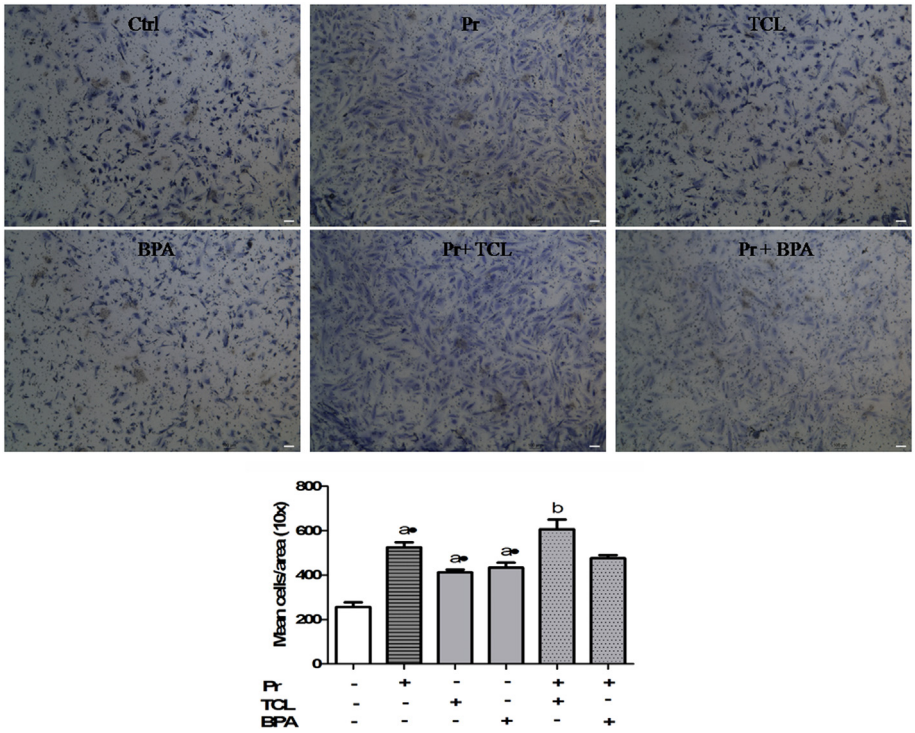

Fig. 7. Migration assay. Representative images of migration assay after TCL or BPA treatment and quantitative evaluation. Pr (10^{-6} M), TCL and BPA (10^{-8} M), enhanced migration of ESCs. ESCs pre-treated for 24 h with Pr and treated with TCL showed the highest number of migrated cells. Scale bar: 100 μm. a, response significantly different than the vehicle control ($P < 0.05$); a•, response significantly different than the vehicle control ($P < 0.01$); b, response significantly different than Pr alone ($P < 0.05$).

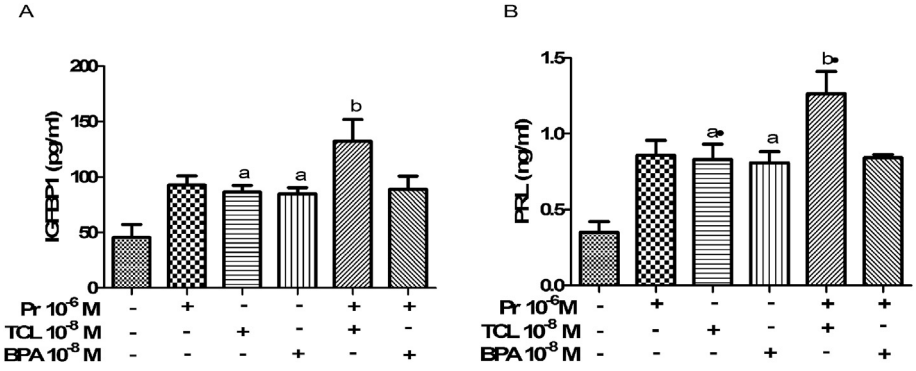

Fig. 8. IGFBP1 and PRL secretion after TCL or BPA treatment. TCL or BPA (10^{-8} M) treatment for 48 h increases the IGFBP1 production (**A**). TCL after 24 h of pre-treatment with Pr (10^{-6} M) showed higher IGFBP1 levels compared to Pr cells. PRL secretion was enhanced after TCL, BPA and Pr treatment. Pre-treatment with Pr induced PRL secretion in ESCs treated with TCL (**B**). a, response significantly different than the vehicle control ($P < 0.05$); a•, response significantly different than the vehicle control ($P < 0.01$); b, response significantly different than Pr alone ($P < 0.05$); b•, response significantly different than the vehicle control ($P < 0.01$).

IGFBP1 at gene and protein level only at 5×10^{-5} M, with no additive effect in ESCs pre-treated with cAMP. Moreover, they did not find variation at the lowest concentration tested (5×10^{-6} M) both for *IGFBP1* and *PRL*. In the same study, authors demonstrated that BPA act in ESCs in a pathway that not involves estrogen receptors. The incongruity between our results and those of Aghajanova and Giudice can be explained surely by the different concentrations of BPA used. Recently the concept of "low dose effects" has been accepted as a general feature for EDCs. The concentrations used in this study are the same previously described in the serum of healthy subjects, both for BPA and TCL (Takeuchi and Tsutsumi, 2002; Allmyr et al., 2008). In summary, our results show that TCL or BPA might trigger decidualization even in absence of other decidualization stimuli. However, when progesterone is present, these mechanisms appear to be additive. Finally, we analyzed the action of TCL or BPA on the migratory capacity of ESCs. Both EDCs, as well as progesterone, strongly induce ESCs to migrate. Expression of genes involved in migration resulted deregulated especially in ESCs exposed to TCL, compared to other treatments. Specifically, 10^{-8} M TCL seems to induce migration through up-regulation of *MMP3* and *MMP9* and via down-regulation of *TIMP1*, *TIMP2*. The ability to potentiate the migration induced by Pr probably involves a *MMP3-MMP9-TIMP2* dependent pathway. BPA at 10^{-8} M, instead, induced ESCs migration via *MMP3* and *MMP9* without affecting TIMP expression. Migration of cells belonging to endometrium stromal compartment could be considered as a clear signal of differentiation, and it has been demonstrated that expression of metalloproteases, the keys molecules for cell migration, increases

in stromal cells during decidualization (Gellersen et al., 2010; Guo et al., 2011).

5. Conclusions

This study strongly suggests that some EDCs act on human stromal compartment of endometrium. Our results clearly indicate that TCL and BPA are able to interfere with molecular pathways of decidualization, enhancing the expression of some key molecules involved in this process and to stimulate stromal cells cell migration. Notwithstanding the molecular pathways involved in the action of TCL or BPA on stromal endometrial cells need further investigations, translating this data to humans, it is possible to speculate that exposure to TCL or BPA may be considered a risk factor for the endometrial physiology, which in turn leads to reproductive failure. Human are routinely exposed to BPA and TCL but in women, by mimicking steroid hormones, EDCs may alter the normal equilibrium in progesterone and estradiol homeostasis, so creating a receptive uterine environment which decreases the probability to gain a successful embryo implantation. Moreover, our results indicated that low concentration of EDCs, might have significant biological effects on humans. Finally, in this study we highlighted the deleterious effects of TCL, an EDC less known in literature but usually found in personal care products.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2015.11.017.

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