Gene expression is altered after bisphenol A exposure in human fetal oocytes in vitro

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Submitted on September 20, 2011; resubmitted on November 8, 2011; accepted on November 16, 2011

ABSTRACT: Bisphenol A (BPA) is a ‘weak’ endocrine disruptor. The effect of BPA on human reproduction is controversial but has been related to meiotic anomalies, recurrent spontaneous abortion, abnormal karyotypes, the diminishing of oocyte survival, delay in meiotic progression and an elevated rate of MLH1 foci in vitro. The aim of this study is to characterize the gene expression of human fetal oocytes in culture as well as to evaluate the effect of BPA in cultured human oocytes. To accomplish our objective, 12 ovaries from 6 euploid fetuses were used. The ovarian fetal tissue was cultivated in two groups: control group and BPA group (BPA30 μM). The cultures were analyzed at T0 and after 7 (T7), 14 (T14) and 21 (T21) days of culture. Evaluation of gene expression was performed by real-time PCR (RT–PCR), with the evaluated genes being: Smc1β, SyCP1 (pairing-synapsis), Spo11, Rpa, H2Ax, Mlh1 and Blm [double-strand break (DSBs) generation, signaling and repair], Era, Erβ and Erγ (estrogen receptors), Stra8 and Nalp5 (markers of meiotic progression). Oocytes from ovaries cultured and treated with BPA show changes in the expression of Spo11, H2Ax and Blm genes, with a significant increase from 3- to 5-fold (P ≤ 0.05). Finally, Rpa, showed a 100-fold increment (P ≤ 0.01). Era, Erβ and Erγ genes showed a BPA up-regulation of 2–4-fold in all of the culture times (P ≤ 0.05). Oocytes exposed to BPA showed an up-regulation of genes involved in DSB generation, signaling and repair except by Mlh1. Thus, BPA can modify the gene expression pattern, which may explain the effects of BPA on female germ cells.

Key words: gene-expression / human / oocyte / BPA / meiosis

Introduction

In almost all female mammals, including the human, meiosis starts during in utero development and only concludes when the oocyte is fertilized. Meiosis is a special kind of cell division characterized by the reductional division of the genome, generating haploid cells after two successive cell divisions following one round of DNA replication. During fetal life, the first meiotic prophase occurs, and then oocytes remain arrested at dictyonema for years or decades. Some important events occur during the meiotic prophase such as meiotic pairing-synapsis and recombination of homologous chromosomes, these processes starting with the generation of double-strand breaks (DSBs) along the genome. DSBs could be repaired by homologous recombination either as crossovers (COs, when the DSB-flanking regions are exchanged between the homologs) or as non-crossovers (NCOs, when no exchange happens between the homologs). When the exchange between homologous chromosomes is performed, mismatch repair-protein MLH1 indicates the places where meiotic recombination (crossovers) occurs (Hoffmann and Borts, 2004). Bisphenol A [BPA; 2, 2-bis (4-hydroxyphenyl) propane; CAS# 80-05-7] is one of the most widely produced chemical products around the world (Vandenberg et al., 2007). This compound is used in the production of polycarbonate plastics and epoxy resins found in metal cans and many other plastic products (toys, drink containers, food packing, eyeglass lenses, dental sealants and medical equipment; Vandenberg et al., 2009, 2010a, b). The most common way of exposure in the human is by oral intake. Exposure of the plastics to high temperatures during boiling, microwave cooking or autoclaving causes migration of the compound from canned beverages, foods and baby bottles (Biles et al., 1999; Imanaka et al., 2001; Goodson et al., 2004; GuoBing et al., 2005; Onn Wong et al., 2005; Wetherill et al., 2007; Chapin et al., 2008; Willhite et al., 2008). Exposure to BPA may also occur by workplace-related inhalation during its production (Vandenberg et al., 2010a, b).
BPA is a ‘weak’ endocrine disruptor with an estrogenic effect. The effect of BPA is mediated by the union of the compound to ERα and ERβ, but BPA has a higher affinity to ERβ (10 times more affinity; Kuiper et al., 1998; Pennie et al., 1998), and it could also activate the cell-membrane estrogen-related receptor γ (ERRγ; Horard and Vanacker, 2003; Okada et al., 2008). Reproductive health effects of BPA on humans are controversial, but BPA-exposure has been related to obesity, endometrial hyperplasia, recurrent spontaneous abortion, abnormal karyotypes and polycystic ovarian syndrome (Takeuchi and Tsutsumi, 2002; Takeuchi et al., 2004; Sugira-Ogasawara et al., 2005; Yang et al., 2006). BPA has been measured in human amniotic fluid, fetal plasma and the placenta, indicating that BPA can cross the placental barrier (Schonfelder et al., 2004; Schonfelder et al., 2004; Schonfelder et al., 2004) and affect fetal metabolism, increasing the possible toxic effect of BPA on all fetal organs (Schonfelder et al., 2002; Zalko et al., 2003; Schonfelder et al., 2004; Engel et al., 2006; Kuruto-Niwa et al., 2007; Mariscal-Arcas et al., 2009).

Fetal ovaries, and thus developing oocytes, exposed to BPA shows different effects during meiosis. Effects of BPA on mouse late-stage oocytes are retardation to reach metaphase I, telophase I arrest, elongation of the spindle and incapacity to resume meiosis. Mouse oocytes exposed to BPA fail to properly align chromosomes at the spindle equator at metaphase II, they present unbalanced chromosome sets and pups obtained from exposed oocytes have the highest rates of abortion (Hunt et al., 2003; Can et al., 2005; Eichenlaub-Ritter et al., 2008; Lenie et al., 2008; Mlynarcikova et al., 2009). Mouse oocytes exposed during fetal development (in utero) have shown that BPA alters synopsis and meiotic recombination (Hunt et al., 2003). Mouse BPA-exposed oocytes also showed increased levels of the MLH1 foci and chiasmata (Hunt et al., 2003; Susiarjo et al., 2007). The observed effects in mouse were also seen in Caenorhabditis elegans (C. elegans); worms exposed to BPA had a low number oocytes, a high rate of embryo lethality, abnormal chromosome synopsis and disruption of DSBs repair progression (Allard and Colaiacovo, 2010). BPA-exposed C. elegans had impaired chromosome segregation during metaphase (Allard and Colaiacovo, 2010).

Recently, our group studied the effects of BPA on human fetal oocytes from cultured ovaries (Briñeo-Enríquez et al., 2011). Human fetal BPA-exposed oocytes showed a delay in meiotic progression characterized by increments of the oocytes at leptotene, diminution in the percentage of oocytes that reached pachytene and reduction in the viability of the oocytes in culture. Increments of degenerated oocytes were observed for all of the BPA concentrations applied (from 1 to 30 μM). Human BPA-exposed oocytes also showed a significant increase in the MLH1 foci number (treated oocytes double the normal values). The increments of the MLH1 foci number were associated with the concentration of BPA applied; in this sense, oocytes cultured with 10 μM BPA or higher showed this effect (Briñeo-Enríquez et al., 2011).

Global gene expression of human fetal ovaries was described by Houmard et al. (2009). The authors reported a temporal change in the gene expression of transcripts involved in meiosis. To date, studies have revealed that the temporal appearance in pairing-synapsis and recombination proteins, in the 12th week of gestation, the expression of Sycp3, Stra8, Stag3, Text1, Text14 and Spo11 increases and the levels of expression continues until Week 22 (last gestational age analyzed; Houmard et al., 2009). Genes known to be involved in meiosis are highly expressed in the mouse ovary, including Sycp1, Msh4 and Msh5, and Dmc1 (Lawson et al., 2010). Other genes associated with meiosis include Spo11, Stag3, Sycp3, Text1l and Text14 (Lawson et al., 2010). Notably, a 100-fold increase in Stra8 was demonstrated in the mouse fetal ovary coincident with the onset of meiosis (14.5 dpc), and a 50-fold increase in Nalp5 was observed when oocytes progressed to the primordial follicle (Fowler et al., 2009; Lawson et al., 2010).

Previous studies described that BPA could affect genes involved in differentiation, cell growth, apoptosis and DNA repair (Naciiff et al., 2002; Yamada et al., 2002; Lemmen et al., 2004; Daftary and Taylor, 2006; Iso et al., 2006; Miyamoto et al., 2006; Iso et al., 2007). In this sense, this compound could affect gene expression of the ovary and uterus. In fact, the effect of BPA on the expression of genes involved in mouse meiosis was studied by Lawson et al. (2010). The authors described a significant change in gene expression in the fetal ovaries of BPA-exposed fetuses after 24 h. Genes related to meiosis showed an up-regulation after BPA exposure but only Msh4, Dmc1 and Sycp2 were statistically different from the control group. However, almost all studies have involved exposures to rodents and these results cannot be generalized to humans; a typical example is diethylstilboestrol (DES) that, in rodents, had no effect, but in humans causes vaginal cancer (Honma et al., 2002; Suzuki et al., 2002). One of the most significant concerns is the suggestion that some of the effects resulting from prenatal and early post-natal exposure to endocrine disruptors are multi-generational; that is, exposure to one generation increases the likelihood of defects in subsequent generations. Multi-generational effects have now been described in mouse studies of DES, BPA and mixtures of vinclozolin with methoxychlor (Imanaka et al., 2001; Goodson et al., 2002; Engel et al., 2006; Yang et al., 2006). Although this mechanism currently is thought to be the result of epigenetic changes that are inheritable, data remain limited.

The aims of this in vitro study are to evaluate, for the first time, the effects of BPA on gene expression of human fetal oocytes during meiotic prophase. To accomplish our objective, genes involved in meiotic pairing-synapsis (Smc1β, Sycp1), DBS generation, signaling and repair (Spo11, Rpa, H2ax, Mlh1 and Bim), markers of meiotic progression (Stra8 and Nalp5) and ERs (Era, Erβ and Eγγ) were analyzed in control-medium and BPA-exposed cultured oocytes. Evaluation of gene expression was performed by real-time PCR (RT–PCR). Quantitative gene expression was normalized to values obtained in fresh oocytes (T0) and as an internal control ribosomal sub-unit 18s (h18s) were used.

### Materials and Methods

#### Biological material

Twelve ovaries from six fetuses were obtained from the Vall d’Hebron Fetal Tissue Bank, following the rules of the Ethical Committee of the Hospital de la Vall d’Hebron, Barcelona, Spain. Last menstrual period, ultrasound analysis and foot length were used to calculate gestational age. All of the fetuses were euploid, the karyotype was obtained by prenatal diagnosis and confirmed in the laboratory with the karyotype of cultured fetal ovary stromal fibroblasts (Roig et al., 2003). Inclusion criteria were euploid fetuses, gestational age between 18 and 22 weeks, <2 h after...
the end of the obstetric procedure and the start of the culture process. Cases used are shown in Table I.

**Culture of human fetal oocytes**

Ovaries were collected and transported to the laboratory at 4°C in DMEM (Dulbecco’s Modified Eagle Medium; Gibco BRL) supplemented with 100 IU/ml penicillin and 50 μg/ml streptomycin (both from Gibco BRL). Ovaries were cultured following the fetal human oocytes culture technique described elsewhere (Brieño-Enriquez et al., 2010). Briefly, to ensure equal inclusion of cortical and medullar ovarian regions, each ovary was cut transversely, obtaining four equal-sized pieces of ~3 x 5 x 5 mm (width x depth x height). The ovary pieces were cut with a McIlwain Tissue Chopper (Ted Pella Inc; Redding, CA, USA) into mini-blocks of ~1 x 1 x 1 mm. After that, the mini-blocks were re-suspended in culture medium. Five mini-blocks per well were seeded in a 24-well cell-culture cluster (Costar, Corning, Inc., NY, USA). The mini-blocks were seeded and cultured for 7 (T7), 14 (T14) or 21 days (T21) at 37°C, 5% CO₂.

Control medium used was DMEM (Gibco BRL) supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 100 ng/ml stem cell factor (SCF; all from Sigma; Munich, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin (both from Gibco BRL). BPA-supplemented medium: control medium ± BPA 30 μM. Bisphenol A was dissolved in 0.01% dimethyl sulfoxide (all from Sigma). Every 2 days, 50% of the culture medium was changed for fresh medium. The oocytes were removed from the wells using (0.25%) trypsin (5 min. at 37°C), and the enzyme reaction was blocked with non-supplemented DMEM medium. The cellular suspension obtained was centrifuged for 10 min at 600g, and immediately thereafter the pellet was re-suspended in 5 ml of PBS and re-centrifuged for 10 min at 600g. Isolated oocytes were used to perform RNA extraction and reverse transcription.

**RNA isolation and reverse transcription**

Total RNA from each culture condition was extracted using the Qiagen mini RNA Easy kit in accordance with the manufacturer’s instructions (Qiagen, Inc., Valencia, CA, USA). Each sample was supplemented with 2 μl of poly-A RNA as a carrier. A DNase step was performed to remove genomic DNA. Total RNA was eluted in 30 μl of RNase free water. RNA concentration was then determined spectrophotometrically by the multi-Detection Microplate Reader (Danippon Sumitomo Pharma Co, Osaka, Japan). From 2 ng of total RNA, cDNA was reverse-transcribed using oligo (dT) and SuperScript III RNase reverse transcriptase (Invitrogen Co, Carlsbad, CA, USA) as was described by the manufacturer. Briefly, synthesis of first-strand cDNA was performed on 2 μl of extracted RNA by adding 0.05 μl of random primers (250 ng/μl), 1.6 μl DNTPs (10 mM) and 16.35 μl of RNase-free water. The mix was heated for 5 min at 65°C and then incubated for 5 min on ice. After that, 4 μl of first-strand buffer 5x, 1 μl of DTT (0.1 M), 1 μl of RNase-OUT (Invitrogen Co) and 1 μl of SuperScript III Reverse Transcriptase (200 U/μl; Invitrogen Co) were added to the original mix. Cycling conditions for reverse transcription were as follows: 5 min at 25°C, 60 min at 55°C and 15 min at 70°C. To remove RNA complementary to the cDNA, 1 μl (2 units) of RNase H (Invitrogen Co) was added to the mix and incubated for 20 min at 37°C.

**Quantitative real-time PCR**

Real-time PCR amplification was performed in a Bio-Rad CFX-Real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) with SYBR-Premix Ex Taq (TakaRa Bio) as previously described. Gene expression was normalized to h18s expression at T0. Standard curves were generated for h18s and all of the genes analyzed, by amplifying serial dilutions of testis cDNA obtained by reverse-transcription from Total Human Testes RNA (Stratagegen). Each reaction mixture consisted of 2.5 μl of cDNA and 22.5 μl of PCR mix containing 0.25 μl of forward primer (0.2 μM), 0.25 μl of reverse primer (0.2 μM), 12.5 μl of SYBR Premix Ex Taq (Takara Bio) and 9.5 μl of nuclease-free water. Quantitative RT–PCR amplification reaction was performed with specific primers as shown in Table II. PCR conditions were as follows: 30 s at 95°C, followed by 45 cycles at 95°C for 1 s and 60°C for 20 s. After PCR, the melting curve analyses were performed to verify specificity and identity of the PCR products. All data were analyzed with the CFX-manager Bio-Rad (Bio-Rad Laboratories). All analyzed genes were performed in triplicate for each gene, culture time and culture media applied.
Table II  Primers used.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
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<tbody>
<tr>
<td>Stra8</td>
<td>CCTCAAGTGCGGAGTTTCTGA</td>
<td>TCCTCTAAGTGCGGTCTGCA</td>
</tr>
<tr>
<td>Nalp5</td>
<td>CGAGGTCGTAAGGAGAACCATCTT</td>
<td>CAGCGGCGGGTGAAG</td>
</tr>
<tr>
<td>MhlI</td>
<td>GGGCCTGTGGAATCCTCAGT</td>
<td>TTCCTCTGGCAGCGCCACCT</td>
</tr>
<tr>
<td>Spo1I</td>
<td>ACATTTTCAGGGCTCTCTAGA</td>
<td>TCTATGAGATGGAAAAC</td>
</tr>
<tr>
<td>Rpa</td>
<td>GGATGATACCGACTCGAGAAG</td>
<td>AGTCACCCAGCTTCCCAGATG</td>
</tr>
<tr>
<td>Sycp1</td>
<td>GGTCTTTCCTGGCCCTCACA</td>
<td>TGATCTCGGTGATGAAACA</td>
</tr>
<tr>
<td>Bmn</td>
<td>GCTGGAAGGATTGGAGTTTG</td>
<td>AAATCCACCTGCTGAAACAA</td>
</tr>
<tr>
<td>H2ax</td>
<td>GGCCGCACGGATTTTATCA</td>
<td>GCCTGACGAGGTTACAGAC</td>
</tr>
<tr>
<td>Era</td>
<td>GGAGCTGAAATCCAGAAACC</td>
<td>AGCACGGATCTCATGGTCTC</td>
</tr>
<tr>
<td>Erβ</td>
<td>AGAGACCTGAGAGGAGGAGG</td>
<td>GCTTCACTTTAAAAGGCTC</td>
</tr>
<tr>
<td>Erγ</td>
<td>TGAGATCTTCGACATGCTG</td>
<td>GCCATCGAGTGATGAAAG</td>
</tr>
<tr>
<td>h18s</td>
<td>GTGGAGCCTATGTTGTCTGGT</td>
<td>CGGACATCGCGAGGCA</td>
</tr>
</tbody>
</table>

Statistical analysis

Statistical analysis among sample repeats and inter-sample repeats was performed using the U–Mann–Whitney test. Two-way ANOVA followed by the Bonferroni post-test was applied to compare control oocytes to treated oocytes. Statistical significance for variables in all tests was set at ≤0.05.

Results

Recently, our laboratory reported a new technique that permits meiotic progression in culture (Briño-Enriquez et al., 2010) as well as the cytogenetic effects of BPA in cultured human fetal oocytes (Briño-Enriquez et al., 2011). Thus, to explore the possible molecular mechanisms implied in the BPA effect, 12 ovaries were cultured with control medium and BPA-supplemented medium for 7, 14 and 21 days. As a control to genes shared by meiosis and mitosis, ovarian fetal fibroblasts were cultured under similar conditions to those applied to the oocytes from cultured ovaries. Genes evaluated included transcripts related to cohesion proteins (Smc1β), synapsis proteins (Sycp1), DNA-DSB generation, signaling and repair (Spo11, Rpa, H2ax, MhlI and Bmn), ERs (Era, Erβ and Erγ) and markers of meiotic progression (Stra8 and Nalp5).

Gene expression in cultured human fetal oocytes using control medium

Gene expression of meiotic progression markers Stra8 and Nalp5

Genes used as meiotic progression markers, Stra8 and Nalp5, showed an increment of transcript expression in oocytes from cultured ovaries associated with the times of culture. Stra8 has been related to the entry of oogonia into meiosis, the Stra8 transcript showed a significant increase in transcript expression in relation to days of culture. In this sense, cultured oocytes had 0.4-fold excess at T7, 1.7-fold excess at T14 and 0.42-fold excess at T21 (P ≤ 0.05). The increase in Stra8 could indicate the entry of oogonia into meiosis in culture (Fig. 1A).

Transcript expression of Nalp5, associated with the progression of the oocyte to the primordial follicle, increased in cultured oocytes. The expression increment was also related to time of culture; Nalp5 showed a significant initial reduction in transcript expression at T7 (0.3-fold less; P ≤ 0.5), followed by a significant increase at T14 and T21 (P ≤ 0.5). The increments went from a 1.0-fold excess at T14 to a 1.5-fold excess at T21. The over-expression of Nalp5 in cultured oocytes indicates that oocytes may have progressed to primordial follicles in culture (Fig. 1B).

Cohesion (Smc1β) and synapsis (Sycp1) genes are not modified by culture conditions along different culture times

Previous reports in the fetal ovary showed that gene expressions Smc1β and Sycp1 were not modified by gestational age of the fetus (Houmard et al., 2009). Human fetal oocytes cultured following our technique (Briño-Enriquez et al., 2010) mimic the gene expression pattern described previously by Houmard et al. (2009). Smc1β expression did not show differences among the different culture times (T7, T14 and T21; P ≥ 0.05). These results could indicate that transcript expression was not modified during the culture (Fig. 2A).

SYCP1 is a component of the central element of the synaptonemal complex that appears during meiotic prophase in oocytes. Previous reports have indicated that gene expression of Sycp1 increases after 12 weeks of gestation and remains stable until 24 weeks of gestation (Houmard et al., 2009). Cultured oocytes did not show differences among the different culture times, indicating that gene expression of Sycp1 was not affected by culture conditions (Fig. 2B). When the gene expression values during culture were compared with those observed in un-cultured oocytes (T0), some significant increases could be seen. These increments went from a 1.5-fold excess at T7 to a 1.0-fold excess at T21, all of them being statistically different, when compared with un-cultured oocytes (T0; P ≤ 0.5). The increments of Sycp1 could be related to the entry of the oogonia into meiosis and, in consequence, into the pairing-synapsis process (Houmard et al., 2009; Lawson et al., 2010).

DSB generation (Spo11), signaling (H2ax) and repair (Rpa, Bmn, MhlI) genes are not modified by culture conditions along different culture times

Protein SPO11 is related to the generation of DSBs required for homologous chromosome pairing-synapsis and meiotic recombination. Oocytes from cultured ovaries did not show any changes in Spo11.
expression, when compared with un-cultured oocytes (T0; \( P \geq 0.05 \)). No differences were observed among the different culture times, indicating that culture conditions did not affect gene expression of \( \text{Spo11} \) (Fig. 3A; \( P \geq 0.05 \)).

Protein H2AX is related to the signaling of the areas where DSBs have occurred. Cultured oocytes did not show differences in \( \text{H2ax} \) expression among the different culture times (Fig. 3B; \( P \geq 0.05 \)). Compared with un-cultured oocytes (T0), cultured oocytes showed an increase in the gene expression of \( \text{H2ax} \), the increments being a 1-fold excess at T7, 1.5-fold excess at T14 and a 3-fold excess at T21 (\( P \leq 0.05 \)), indicating that culture conditions could change \( \text{H2ax} \) expression, which is probably secondary to tissue manipulation.

Protein RPA is involved in DSB repair. No differences were observed among different times of culture (Fig. 3C; \( P \geq 0.05 \)). Cultured oocytes showed an increment of gene expression of \( \text{Rpa} \) in all culture times. Increments observed were a 0.8-fold excess at T7, 0.7-fold excess at T14 and 0.6-fold excess at T21 (\( P \leq 0.05 \)). The higher increases were observed at T7 and the decreases at T21, suggesting that \( \text{Rpa} \) over-expression could be related to DNA damage generated by the manipulation of the tissue at the beginning of the culture.

Protein BLM (Bloom helicase) is involved in the DSB-repair process and is shared by meiosis and mitosis. No differences were observed among different times of culture. Cultured oocytes showed a diminishing \( \text{Blm} \) expression, when compared with fresh oocytes. Oocytes at T7 and T14 showed a significant diminution of \( \approx 0.4 \)-fold, as compared with T0 (\( P \leq 0.05 \); Fig. 3D). These results could indicate that in oocytes from cultured ovaries, this mechanism of DSBs is a secondary repair pathway.

Protein MLH1 is a marker of meiotic recombination; oocytes from cultured ovaries did not show differences among the different culture times (T7, T14 and T21; \( P \geq 0.05 \)). Compared with un-cultured oocytes, the statistical reduction of \( \text{Mlh1} \) expression went from 0.4-fold at T0 to 0.3-fold at T14 (\( P \leq 0.05 \); Fig. 3E). These results could indicate that the greatest levels of \( \text{Mlh1} \) transcription occurred probably before the beginning of culture. Previous reports in mouse and human fetuses had reported that values of \( \text{Mlh1} \) were not modified by the gestational age and remained at stable levels from Weeks 12 to 22 (Houmard et al., 2009; Lawson et al., 2010).
**ER genes** (Erα, Erβ and Errγ) are not modified by culture conditions along the different culture times.

ERs are involved in the development of oocytes as well as follicles; in this sense, Erα, Erβ and Errγ expressions were evaluated in oocytes from cultured ovaries. No differences were observed among T7, T14 and T21 for any of the ER genes (P ≥ 0.05). Nevertheless, when cultured oocytes were compared with un-cultured oocytes, a significant diminution in Erα expression was observed in all culture times, with the decrease in Erα expression ranging from 0.2-fold at T7 to 0.5-fold at T21 (P ≤ 0.05; Fig. 4A).

Erβ and Errγ expression increased in all culture times. Erβ expression showed a significant increment of 0.2-fold at T7, 0.6-fold at T14 and 1-fold at T21 (P ≤ 0.05; Fig. 4B). Cultured oocytes showed an increase in Errγ expression, with the range of increase from 0.8-fold at T7 to 0.6-fold at T21, all of them being statistically different to values observed in un-cultured oocytes (Fig. 4C). In this sense, one could argue that culture conditions did not change the gene expression of the genes if they were compared among different times of culture (T7, T14 and T21), but if we compare them to un-cultured oocytes, some modifications could be observed.

**Effects of BPA in gene expression of cultured human fetal oocytes**

Meiotic progression marker (Stra8 and Nalp5) genes are not regulated by BPA

As was described in oocytes cultured with control medium, oocytes cultured with BPA-supplemented medium also showed an increase...
in gene expression of \emph{Stra8} and \emph{Nalp5}, when compared with un-cultured oocytes (T0). In this way, \emph{Stra8} showed an increment of expression after BPA exposure, which were 0.5-fold at T7, 2.0-fold at T14 and 0.8-fold at T21, all of them being statistically different from un-cultured oocytes \((P \leq 0.05; \text{Fig. 1A}).\)

Similarly, \emph{Nalp5} was also over-expressed in cultured BPA-exposed oocytes. The increment of expression was related to time of culture; in this manner after an initial ‘normal’ level of expression at T7, a significant increase was observed at T14 and T21. The increments went from 0.2-fold at T14 to 0.8-fold at T21 \((P \leq 0.05; \text{Fig. 1B}).\) In all culture times \((T7, T14 \text{ and } T21),\) no statistical differences were observed among oocytes cultured with control medium or those cultured with BPA-supplemented medium. Oocytes from cultured ovaries and treated with BPA-supplemented media followed the \emph{Stra8} and \emph{Nalp5} gene-expression pattern observed in those cultured with control medium. These data indicate that, at least in culture, the gene markers of the entry of oogonia into meiosis and the development of the primordial follicle seem to not be regulated by BPA.

Cohesion (\emph{Smc1β}) and synapsis (\emph{Sycp1}) genes are not regulated by BPA

BPA-exposed oocytes from cultured ovaries did not show differences between oocytes cultured with control medium at the different culture times \((T7, T14 \text{ and } T21; P \geq 0.05).\) These results could indicate that the \emph{Smc1β} gene expression was not modified by BPA-exposure. A diminution in \emph{Smc1β} expression at all culture times was observed when it was compared with values observed in un-cultured oocytes. The observed significant reduction in gene expression was \(\sim 0.3\)-fold less at T7, T14 and T21, when compared with T0 \((P \leq 0.05; \text{Fig. 2A}).\) Cohesins are synthesized before the beginning of meiosis, so the reduction could be related to culture conditions.

\emph{Sycp1} expression was increased in oocytes cultured with BPA-supplement, as compared with un-cultured oocytes \((T0; P \geq 0.05).\) Oocytes cultured with BPA-supplemented medium showed an increment of 1.3-fold excess at T7, 1.4-fold excess at T14 and 0.9-fold excess at T21, all of them being significantly different from T0 \((P \leq 0.05; \text{Fig. 2B}).\) In contrast, no differences between oocytes cultured with control medium or BPA-supplemented medium were observed for any of the culture times. These results could suggest that BPA did not regulate \emph{Sycp1} expression.

BPA up-regulated the expression of genes involved in DSB generation (\emph{Spo11}), signaling (\emph{H2ax}) and repair (\emph{Rpa}, \emph{Bml})

BPA-exposed oocytes showed an increment of gene expression of \emph{Spo11}, \emph{H2ax}, \emph{Rpa} and \emph{Bml}, when it was compared with un-cultured oocytes \((T0)\) and to oocytes cultured with control medium. \emph{Spo11} increments in BPA-exposed oocytes went from a 0.5-fold excess at T7 to a 1.5-fold excess at T21, all of them being statistically different from un-cultured oocytes \((T0; P \leq 0.05; \text{Fig. 3A}).\) The same trend was observed when oocytes cultured with BPA-supplemented medium...
were compared with those cultured with control medium, the observed increments are from a 0.7-fold excess at T7 to a 1.1-fold excess at T21 (P ≤ 0.05), indicating that BPA could induce DSBs by the increase of Spo11 expression.

Up-regulation of H2ax in BPA-exposed oocytes was also observed. Compared with un-cultured oocytes, BPA-exposed oocytes showed a significant increment of expression, with ranges from a 5.5-fold excess at T7 to a 10.9-fold excess at T21 (P ≤ 0.05; Fig. 3B). Up-regulation of H2ax by BPA was also observed, when compared with oocytes cultured with control medium. Observed increments went from a 4.4-fold excess at T7 to an 8.7-fold excess at T21, all of them being statistically significant (P ≤ 0.05). Increments in DSBs were generated by Spo11, so the over-expression of H2ax could be related to the activation of repair pathways, after Spo11 activity on DNA.

Rpa is related to the repair of DSBs; oocytes cultured with BPA-supplemented medium showed an up-regulation of Rpa, when compared with un-cultured oocytes and to oocytes cultured with control medium. For both, uncultured oocytes as well as oocytes cultured with control-medium, significant increments of a 24.9-fold excess at T7, 30.5-fold excess and 98.5-fold excess at T21 were observed in BPA-exposed oocytes (P ≤ 0.05, P ≤ 0.01 and P ≤ 0.001, respectively; Fig. 3C). As a consequence of the increments of DSB generation, the oocytes need to repair DNA. In this sense, the cells induce over-expression of Rpa to try to maintain the DNA structure.

Blm gene expression did not show differences, when compared with fresh oocytes, but when these oocytes were compared with oocytes cultured with control medium an increase from a 0.3-fold excess to a 0.6-fold excess was observed. The up-regulation of Blm by BPA was only statistically significant at T14 (P ≤ 0.05; Fig. 3D). These observations could indicate that Blm is not a main pathway of DSB repair in oocytes in culture.

Meiotic recombination marker gene (Mlh1) is not regulated by BPA

Previous studies in mouse and human (Susiarjo et al., 2007; Lawson et al., 2010; Briñüño-Enriquez et al., 2011) have described an increment of meiotic points marked by MLH1 in BPA-exposed oocytes. The Mlh1 gene expression of oocytes from ovaries cultured with control medium and BPA-supplemented medium was analyzed. Mlh1 was down-regulated in all conditions tested, the ranges of decrease went from 0.4-fold less at T7 to 0.3-fold less at T21 (Fig. 3E). Statistical differences were observed at T7 and T14 for both culture media. Surprisingly, after 21 days of culture, the values of Mlh1 expression were closer to the values observed in un-cultured oocytes (T0).

Erα, Erβ and Errγ are up-regulated by BPA

Oocytes from ovaries cultured with BPA-supplemented medium showed an up-regulation of Erα, when compared with un-cultured oocytes as well as oocytes cultured with control medium. The range of increments went from a 0.1-fold excess at T7 to a 0.31-fold excess at T21 (Fig. 4A). Only at T14 and T21 were the increments statistically significant (P ≤ 0.05). Differences in gene expression were also significant at T14 and T21 in oocytes cultured with BPA-supplemented medium, as compared with those cultured with control medium (P ≤ 0.05).

Erβ is highly expressed in fetal human oocytes at 20 weeks of gestation; oocytes from ovaries exposed to BPA in culture showed a significant increment of a 1.5-fold excess at T7, 2.3-fold excess at T14 and 2.9-fold excess at T21, when compared with un-cultured oocytes (P ≤ 0.01; Fig. 4B). BPA-exposed oocytes showed a statistical increase from a 1-fold excess at T7 to a 2-fold excess at T21 to control-medium cultured oocytes (P ≤ 0.01; Fig. 4B).

Finally, BPA action has been related to ERRγ activation (Takayanagi et al., 2006; Okada et al., 2008); in this sense, oocytes from ovaries cultured with control medium showed an increment of Errγ, but the increments observed in BPA-exposed oocytes were significantly greater. BPA-exposed oocytes showed significant increments of Errγ expression, from a 2.2-fold excess at T7 to a 4.3-fold excess at T21, as compared with un-cultured oocytes (P ≤ 0.001; Fig. 4C). Differences between control medium and BPA-supplemented medium were also observed, with BPA-exposed oocytes showing significant increments of Errγ expression. The increments went from a 1.3-fold excess at T7 to a 3.6-fold excess, as compared with oocytes cultured with control medium (P ≤ 0.01).

Genes involved in DSB signaling (H2ax) and repair (Rpa, Blm, Mlh1) are up-regulated in cultured human fetal fibroblasts exposed to BPA

As was mentioned above, some of the genes involved in meiosis are shared with mitosis. Thus, the gene expression was evaluated for these genes in fibroblasts from ovarian stromal tissue and fallopian tubes. The oocyte culture method used involves the use of mini-blocks of fetal ovary and in these blocks fibroblasts could be observed. In order to evaluate if the values of gene expression from cultured oocytes were affected by a possible fibroblast contamination, gene expression of fibroblasts obtained from fallopian tubes and ovarian stromal tissue was evaluated.

Fibroblasts cultured with control medium showed an increment of H2ax expression, from a 1.2-fold excess at T7 to a 2.3-fold excess at T21, all of them being statistically different from un-cultured fibroblasts T0 (un-cultured fibroblasts obtained from fallopian tubes; P ≤ 0.05). Fibroblasts cultured with BPA-supplemented medium showed a significant increase in ranges of H2ax expression, as compared with fibroblasts at T0, with the increases ranging from a 5.6-fold excess at T7 to a 10.9-fold excess at T21 (P ≤ 0.01; Fig. 5A). Statistically significant differences were also observed when fibroblasts cultured with control medium were compared with fibroblasts cultured with BPA-supplemented medium (P ≤ 0.05; Fig. 5A).

The same trend was observed for Rpa; fibroblasts cultured with control medium show an up-regulation in culture. Statistically significant increments were observed at all of the culture times, from a 0.8-fold excess at T7 to a 7.6-fold excess at T21 (P ≤ 0.05; Fig. 5B). BPA-exposed fibroblasts also showed statistically significant increments, with a range of 24.9-fold excess at T7 to a 140.5-fold excess at T21 (P ≤ 0.001). Statistical differences were observed between fibroblasts cultured with control medium and those cultured with BPA-supplemented medium (P ≤ 0.05; Fig. 5B).

Blm was down-regulated in fibroblasts cultured with control medium, as compared with fibroblasts at T0, but differences between them were not significant. Fibroblasts cultured with BPA-supplemented medium showed an increment of Blm expression at T14 and T21, when compared with fibroblasts at T0 (P ≤ 0.05; Fig. 5C). Statistical differences were observed between fibroblasts
cultured with control medium and those cultured with BPA-supplemented medium at T14 and T21 \((P \leq 0.05; \text{Fig. } 5C)\).

As was described, in oocytes, the \textit{Mlh1} gene was down-regulated in cultured fibroblasts. \textit{Mlh1} was also down-regulated in all conditions tested (Fig. 5D), except by BPA-exposed fibroblasts at T21. BPA-exposed fibroblasts at T21 showed a significant up-regulation, with an increment of gene expression of a 0.9-fold excess, as compared with T0 and control medium \((P \leq 0.05)\).

\textit{Erα}, \textit{Erβ} and \textit{Errγ} are up-regulated by BPA in cultured human fetal fibroblasts

Fibroblasts cultured with control medium did not show statistically significant differences, when compared with T0 in \textit{Erα} expression. Fibroblasts cultured with BPA-supplemented media showed an up-regulation, as compared with T0, with increments in gene expression from a 2.4-fold excess at T7 to a 3.4-fold excess at T21, all of them being statistically different \(( P \leq 0.05; \text{Fig. } 6A)\). Differences between fibroblasts cultured in control medium and BPA-supplemented medium were observed at T7, T14 and T21 \(( P \leq 0.05; \text{Fig. } 6A)\).

For \textit{Erβ}, no statistical differences were observed among fibroblasts at T0, as compared with fibroblasts cultured with control medium. Fibroblasts cultured with BPA-supplemented medium showed an up-regulation of \textit{Erβ}, as compared with T0 and fibroblasts cultured with control medium. The significant increments went from a 1.2-fold excess at T7 to a 2.0-fold excess at T21 \(( P \leq 0.05; \text{Fig. } 6B)\).

Finally, the gene expression of \textit{Errγ} was also up-regulated by BPA. Fibroblasts cultured with control medium did not show statistical differences, when compared with T0. BPA-exposed fibroblasts showed an up-regulation, with increments ranging from a 3.2-fold excess at T7 to a 3.3-fold excess at T21, all of them being statistically different from T0 \(( P \leq 0.05; \text{Fig. } 6C)\).

Discussion

Recently, we reported the cytogenetic effects of BPA exposure in cultured human fetal oocytes. BPA-exposed oocytes showed lower viability in culture, a delay in meiotic progression and increased levels of meiotic recombination (Brieñó-Enriquez et al., 2011), so our group decided to evaluate gene expression during prophase in BPA-exposed cultured oocytes. This work shows, for the first time, the gene expression of human fetal oocytes in culture and gene expression of BPA-exposed oocytes. Oocytes from ovaries exposed to BPA showed an up-regulation of genes involved in DSB generation, signaling and repair as well as ERs.

Cultured oocytes follow the time-progression gene expression of human uncultured fetal oocytes

Global gene-expression studies via microarray analysis have a critical role in furthering our understanding of gonadal function before and
after birth. With the generation of global gene-expression data sets comes the immediate desire to perform direct comparisons between species. Improvements in technology have alleviated the need for the copious amount of RNA that was originally required to complete such a study. Previous studies performed on fetuses of mouse and human (Houmard et al., 2009; Lawson et al., 2010) revealed the relationship between gestational age and gene expression. In this manner, genes used as meiotic progression markers, such as \textit{Stra8} and \textit{Nalp5}, showed an increment in gene expression with time in culture. Previously, we had demonstrated that oocytes in culture progress along meiosis in culture, with increments in the percentage of oocytes at leptonema and pachynema (Brien˜o-Enriquez et al., 2010). In this way, the increments of oocytes in the culture could be related to ‘new’ oogonia that initiate meiosis. \textit{Stra8} has been related to the entry of oogonia into meiosis, so if we observe an up-regulation of \textit{Stra8} expression during the culture, it could indicate the entry of these ‘new’ oogonia into meiosis in vitro (Houmard et al., 2009; Tedesco et al., 2009; Lawson et al., 2010). An important observation needs to be clarified, and this is the fact that the increments of \textit{Stra8} reach their highest levels at T14 and then decrease, in opposition to \textit{Nalp5}, associated with progression of the oocyte to the primordial follicle, which reaches the highest levels at T21. These observations indicate that, firstly, a larger number of oocytes could enter into meiosis in culture, and many of these oocytes progress and could form a primordial follicle.

\textit{Smc1}β gene expression was not affected by culture time, indicating that the highest levels of gene expression of \textit{Smc1}β could be before the beginning of the culture, as was observed in uncultured oocytes. It is important to mention that \textit{SMC1}β is supposed to be loaded onto the chromosomes by the time of DNA replication, during the pre-meiotic S phase. For \textit{Sycp1}, an increment was observed in gene expression during the different culture times and was compared with uncultured oocytes. Increments of \textit{Sycp1} gene expression could be related to the entry of oogonia into meiosis because \textit{SYCP1} is necessary to accomplish meiotic synapsis.

On the other hand, genes involved in the DSB generation (\textit{Spo11}), signaling (\textit{H2ax}) and repair (\textit{Rpa}, \textit{Bml}, \textit{Mlh1}) were not modified by culture time, in agreement with previous reports in mouse and human fetuses (Houmard et al., 2009; Lawson et al., 2010). ERs are involved in the development of oocytes as well as follicles. Cultured oocytes showed a diminution in gene expression of \textit{Erα} and a over-expression of \textit{Erβ} and \textit{Err}. Previous reports showed that the presence of ERs in the fetal ovary is related to gestational age. \textit{Erβ} is expressed by the human ovary before Week 20, but \textit{Erx} is only observed after Week 20, so the diminishing in \textit{Erα} could be related to the gestational age of the ovaries cultured as well as to the entry of new oogonia into meiosis (Couse et al., 1999; Vaskivuo et al., 2005).

**Figure 6** Gene expression of estrogen receptors in human fetal fibroblasts in oocytes cultured with the control medium and BPA-supplemented medium. **Statistically different, as compared with uncultured fibroblasts (two-way ANOVA, Bonferroni post-test, \(P \leq 0.05\)). *Statistically different, as compared with the control medium (two-way ANOVA, Bonferroni post-test, \(P \leq 0.05\)).**
of DSBs in human breast adenocarcinoma cells after exposure to BPA (Iso et al., 2006) and an increase in DSB markers, such as γH2AX, which co-localized with DNA-repair proteins such as BLM (Rockmill et al., 2003; Wu and Hickson, 2003; Iso et al., 2006; Iso et al., 2007). Our results show that genes involved in DSB generation, signaling, and repair were up-regulated in BPA-exposed cultured oocytes, in agreement with previous studies (Celeste et al., 2002; Iso et al., 2006; Rodrigue et al., 2006; Myers et al., 2009; Oakley and Patrick, 2010). The increments of Spo11, Rpa and H2ax expression could explain the increases in recombination observed in the cytogenetic analyses, except by Mlh1.

MLH1 is considered the marker of chromosomal recombination, and in cultured oocytes exposed to BPA the number of MLH1 foci duplicates the values described for control oocytes (Briñño-Enríquez et al., 2011). We expected to find an up-regulation of Mlh1 by BPA, but this was not observed. The discussion is still open as the cytogenetic results are not in agreement with gene-expression results. We believe that, probably, the highest levels of gene expression and synthesis of all proteins related to DNA-damage repair, including MLH1, is previous to Week 18. In this sense, the oocyte could have some kind of protein reservoir. When BPA damage is produced, the protein is used by the oocyte. In this way, more studies need to be performed to try to understand the cell pathways involved.

**BPA up-regulated Era, Erβ and Erry in human fetal oocytes in culture**

The effect of BPA has been related to its activity as an ERβ antagonist (Hunt et al., 2003; Susiarjo et al., 2007; Hunt et al., 2009; Allard and Colaiacovo, 2010). Oocytes cultured with BPA-supplemented medium showed an increment of the gene expression of all estrogenic receptors. The up-regulation of all ER genes could be related to an imbalance in the intracellular responses, in this manner the blocking of the ERβ by BPA could cause a feedback positive for ERα and ERRγ. The cell will try to synthesize more receptors as a response to the inactivity of ERβ caused by the antagonist action of BPA, and gene expression could be increased. BPA is the ‘natural’ ligand of ERRγ, the positive signal induces an over-expression of the receptor, as was described in placenta (Horard and Vanacker, 2003; Huppunen and Aarnisalo, 2004; Bouskine et al., 2009; Avissar-Whiting et al., 2010). More studies definitely need to be performed to establish the relations between BPA and the ERs in human fetal oocytes.

**Up-regulation of H2ax, Rpa and Bml by BPA in human fetal fibroblasts**

BPA-exposed fibroblasts showed an up-regulation of H2ax, Rpa and Bml. The observed effect is in agreement with previous studies performed in fibroblasts of different organs (Washington et al., 2001; Lehmann and Metzler, 2004; Willhite et al., 2008; Allard and Colaiacovo, 2010; Avissar-Whiting et al., 2010). As was mentioned above, these genes are shared in mitosis and meiosis; up-regulation performed by BPA indicates the possible regulatory effect the compound has over these genes in both kinds of cell division. The differences observed between the cultures of oocytes and fibroblasts could not explain the cellular pathways involved in the effect of BPA, but it evidently shows its effect on cell division. In fact, the over-expression of Rpa was higher in fibroblasts than in oocytes, indicating that BPA could probably induce a greater effect on somatic cells than on germinal cells.

**Conclusions**

Bisphenol A induces the over-expression of Spo11, H2ax, Rpa and Bml in human fetal ovarian cultures. All of these genes are involved in the DSB generation, signaling and repair during meiosis, but some of them are shared with mitosis (H2ax, Rpa and Bml). The up-regulation caused by BPA could disrupt the cell cycle and, in consequence, cell survival and differentiation. Ovarian pieces exposed to BPA also showed an up-regulation of ERs. Era, Erβ and Errγ, which was also observed in ovarian fibroblasts, suggesting that these receptors could be related to the effects of BPA on the ovary.

The results obtained show the effects of BPA on the fetal ovary as an organ, since most likely both oocytes and fibroblasts are affected by exposure to BPA in culture. Nevertheless, it is important to remember that at the end of meiotic prophase, oocytes establish a close relationship with the ovarian stromal cells to form the primordial follicles, which are the source for gamete precursors in the female. Therefore, effects of BPA on the stromal tissue cells have to be considered when studying the effect of BPA on ovarian function. Thus, either by a direct effect on the oocytes or by an indirect effect on the stromal ovarian cells, or the combination of both, BPA can change the expression pattern of some genes involved in meiotic processes, which may explain the already-known effects of BPA on female germ cells (Wu and Hickson, 2003; Dafty and Taylor, 2006; Houmard et al., 2009; Allard and Colaiacovo, 2010; Lawson et al., 2010; Briñño-Enríquez et al., 2011).

**Authors’ roles**

M.G.C. had full access to all of the data in the study and assumes responsibility for the integrity of the data and the accuracy of the data analysis. M.B.-E. and M.G.C. contributed in the study concept and design, analysis and interpretation of data. M.B.-E., R.R.-V., N.T. and L.C. contributed in the acquisition of data. M.B.-E., I.R. and G.C. in the drafting of the manuscript. M.B.-E., I.R., F.M. and G.C. contributed in the critical revision of the manuscript for intellectual content.

**Acknowledgements**

This work would have been impossible without the support of the clinicians and biologists of the Fetal Tissue Bank, Pathology and
Gynecology Departments of the Hospital de la Vall d’Hebron, Barcelona, Spain, as well as all of the members of the Department of Physiology and Pharmacology of the Medicine School of the Universidad Autónoma de San Luis Potosí, México, members of the Unit of Cell Biology and Medical Genetics of the Medicine School of the UAB, Barcelona, and Dr. Anna Barceló from, the Molecular Biology Department of IBB, UAB. This manuscript was proofread by Mr. Chuck Simmons, a native English-speaking Instructor of English of the Universitat Autònoma de Barcelona.

**Funding**

Financial support for this study was provided by grants from the Ministerio de Educación y Cultura (BFU2006-12951, Spain), CIDEM (ACCIó) Generalitat de Catalunya (Spain), Generalitat de Catalunya (2009 SGR 1107, Spain), Ministerio de Asuntos Exteriores y Cooperación Internacional (AECI, Spain, #0000447445), National Council of Science and Technology (CONACYT, México, #166825) and the National Council of Public Education (SEP, México, SEP-2004-CO1-4591).

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