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ORIGINAL ARTICLE *Infertility*

Bisphenol-A and human oocyte maturation *in vitro*

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STUDY QUESTION: Does exposure to bisphenol-A (BPA) affect the maturation of human oocytes *in vitro*?

SUMMARY ANSWER: There was a dose–response association of BPA exposure with altered human oocyte maturation *in vitro*.

WHAT IS KNOWN ALREADY: There is widespread exposure of the general population to BPA. BPA has been detected in the human follicular fluid. Animal studies have shown that BPA exposure is associated with maturation arrest and spindle abnormalities in maturing oocytes.

STUDY DESIGN, SIZE, DURATION: A randomized trial, using 352 clinically discarded oocytes from 121 patients.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The study population was drawn from patients undergoing IVF/ICSI cycles in our program at Brigham and Women's Hospital from March 2011 to April 2012. Oocytes from only one cycle for each patient were included in the study. Cycles with at least two germinal vesicle stage oocytes were included with random allocation of one oocyte to culture for 30 h without BPA and remaining sibling oocytes to medium-containing BPA (20, 200 ng/ml or 20 μg/ml). Oocytes were fixed and labeled for tubulin, actin and chromatin and examined with immunofluorescence and confocal microscopy. Oocytes were assessed for meiotic stage ($n = 292$), and those at metaphase II (MII, $n = 175$) were further classified according to their spindle configurations and patterns of chromosome alignment. McNemar's test was used to compare dichotomized maturation status. Generalized estimating equations were used to account for the correlation between oocytes from the same woman and for the spindle analysis.

MAIN RESULTS AND THE ROLE OF CHANCE: As the BPA dose increased, there was a decrease in the percentage of oocytes that progressed to MII ($P = 0.002$) and increases in the percentage of oocytes that were degenerated ($P = 0.01$) or that had undergone spontaneous activation ($P = 0.007$). Among MII oocytes, as the BPA dose increased, there was a significant trend (by test for trend) for a decreased incidence of bipolar spindles ($P < 0.0001$) and aligned chromosomes ($P = 0.02$).

LIMITATIONS, REASONS FOR CAUTION: Although we used sibling oocytes to overcome potential confounders, such as infertility diagnosis and maternal age, additional studies with a larger number of oocytes are required to confirm the present results. Having access only to clinically discarded oocytes, we were limited to evaluating only those oocytes that failed to mature *in vivo* despite having been exposed to gonadotrophin stimulation and the ovulatory trigger of HCG.

WIDER IMPLICATIONS OF THE FINDINGS: To our knowledge, this is the first study investigating the effect of BPA on oocyte meiotic maturation, spindle morphology and chromosome alignment in human oocytes. Together with prior animal studies, the data support the negative influences of BPA on cell cycle progression, spindle architecture and chromosome organization during oocyte maturation. Furthermore, the increased rates of abnormal maturation in oocytes exposed to BPA may be relevant to our understanding of the decrease in fertility reported in the last decades.

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Introduction

Bisphenol-A (BPA) is a widespread industrial compound, which has been shown to alter the normal function of the endocrine system, and so is classified as an endocrine disruptor chemical (EDC) (reviewed by Welshons *et al.*, 2006). Although polycarbonate plastics are inert under normal circumstances, in cases of exposure to UV light, basic and acidic solutions and heat, BPA monomers might leach into the environment. The compound is found in polycarbonate plastics and in a variety of other consumer products (Talsness *et al.*, 2009; Lawson *et al.*, 2011). There is evidence that this compound has adverse effects on animal health (Welshons *et al.*, 2006; reviewed by vom Saal *et al.*, 2007 and Vandenberg *et al.*, 2012) and is also suspected of having adverse effects on human health although available data are limited (Moriyama *et al.*, 2002; Lee *et al.*, 2003; Vandenberg *et al.*, 2007; Caserta *et al.*, 2008; Diamanti-Kandarakis *et al.*, 2009; reviewed by Vandenberg *et al.*, 2012). BPA was detected in urine of more than 90% of participants in the National Health and Nutrition Examination Survey, USA (Calafat *et al.*, 2005; 2008) and has also been found in various human fluids including follicular fluid (Ikezuki, 2002). Among a population of 36 Japanese women, the mean BPA level detected in the follicular fluid was 2.4 $\pm$ 0.8 ng/ml (Ikezuki, 2002). A similar mean concentration has been found among patients undergoing IVF in the Boston, MA, USA area (mean 1.56 ng/ml; $n = 59$), although the upper range was 43 ng/ml (Hauser *et al.*, unpublished data).

There is accumulating evidence that fertility is decreasing among both women and men (Guzick and Swan, 2006; Hamilton and Ventura, 2006). One of the suggested explanations for this decline has been the increasing exposure to environmental chemicals. Clinical studies have shown an inverse correlation between urine/serum concentrations of BPA, the number of oocytes retrieved (Mok-Lin, 2009) and peak estradiol (E_2) levels in women undergoing IVF (Mok-Lin, 2009; Bloom *et al.*, 2011). Moreover, a positive linear dose–response association has been demonstrated between BPA urinary concentrations and implantation failure (Ehrlich *et al.*, 2012a,b). Preliminary data have also shown an inverse correlation between BPA levels and the proportion of mature [i.e. metaphase II (MII)] oocytes among patients undergoing ICSI (Fujimoto *et al.*, 2011).

Exposure to environmental toxicants at several life stages may adversely affect oocyte quality (reviewed by Hunt and Hassold, 2008). Meiotic maturation of the oocyte, which occurs in response to the pre-ovulatory surge of LH, may be particularly susceptible to chemical-induced perturbations. This is the process during which the chromosomes segregate on the meiotic spindle and the cytoplasm ultimately matures in preparation for fertilization. The detection of BPA in human follicular fluid is of particular concern because animal studies have demonstrated that exposure of oocytes to BPA is not only associated with meiotic arrest at the germinal vesicle (GV) stage but also may result in alterations in meiotic spindle structure and perturbations in chromosomal alignment, as well as an increased incidence of aneuploidy (Hunt *et al.*, 2003; Can *et al.*, 2005; Eichenlaub-Ritter *et al.*, 2008; Lenie *et al.*, 2008; reviewed by Hunt and Hassold, 2008). However, to the best of our knowledge, the effect of BPA exposure on meiotic maturation in human oocytes has not been previously investigated.

This study was undertaken as a preliminary investigation to determine whether BPA exposure perturbs meiotic maturation, spindle organization and chromosome alignment in human oocytes *in vitro*.

Material and Methods

The study was approved by the Institutional Review Board of Partners' Healthcare.

Source and collection of oocytes

This study utilized immature (GV stage) oocytes that had been aspirated during oocyte retrieval from patients undergoing IVF/ICSI between March 2011 and April 2012 in our IVF program. All patients had undergone standard ovarian stimulation protocols as previously described (Shah *et al.*, 2011; Machtinger *et al.*, 2012) and included: (i) down-regulation protocols using GnRH agonists, (ii) GnRH antagonists and (iii) poor responder protocols using low-dose GnRH agonist flare or E_2 priming. As GV-stage oocytes are deemed clinically useless, they are typically discarded according to the policy of our IVF program. However, oocytes were only released for this research if patients had signed an informed consent for use of their discarded materials. A minimum of two GV-stage oocytes from a patient (i.e. a sibling pair) were required for study inclusion. Oocytes from only one cycle for each patient were included in the study.

GV-stage oocytes were released from the clinical IVF laboratory for use in this study within 1–2 h of partial cumulus cell disruption and assessment of meiotic stage for ICSI. Oocytes are usually stripped from these surrounding cells during a routine ICSI procedure to assess their maturation status. In cases in which partial stripping definitively revealed that the enclosed oocyte was immature, no further stripping was performed. All the immature oocytes included in this study were enclosed by some residual cumulus/corona radiata cells when initially placed in culture. After culture, there was some loosening of the corona–cumulus cells from most oocytes.

Experimental design

Due to both constraints of working with human oocytes that are limited in availability and our wish to investigate the effect of several concentrations of BPA on oocyte maturation, it was impossible to assess all BPA concentrations simultaneously. Therefore, the study was undertaken in two separate experiments: In the first experiment, the effect of exposure to a high concentration of BPA (20 μg/ml) was compared with control media containing no BPA (see below). This concentration was chosen in order to determine whether BPA exposure had any effect on the maturation of human oocytes *in vitro*. In the second experiment, the effects of lower concentrations of BPA (20 ng/ml and 200 ng/ml) on meiotic maturation were tested. When

only two oocytes from a patient were available, one oocyte was randomized to the control group and the other to medium containing 20 ng/ml BPA. For patients for whom three oocytes were available, the third oocyte was cultured in the presence of 200 ng/ml BPA. The 20 and 200 ng/ml concentrations of BPA were, respectively, *c.*10- and 100-fold higher than the published baseline level of BPA previously measured in human follicular fluid (1–2 ng/ml; Ikezuki, 2002).

Culture system and preparation of BPA solutions

Oocytes were cultured for 30 h at 37°C in a humidified atmosphere of 5% CO_2 in air in 8-well glass slides (LabTek, #1256518, No. 177402; Thermo Fisher Scientific, USA) containing 300 μl of SAGE IVM medium (CooperSurgical, Trumbull, CT, USA) with 0, 20, 200 ng/ml or 20 μg/ml BPA (CAS Number: 80-05-7; Sigma-Aldrich).

The 30 h culture period was selected in order to maximize our ability to identify oocytes at MII. In a preliminary study, we had observed that a shorter culture period (24–27 h) resulted in higher rates of telophase I (TI) oocytes, while after a longer incubation (34–36 h) there was a higher incidence of spontaneous activation. Stock BPA (20 mg/ml for Experiment 1 and 20 μg/ml for Experiment 2) was dissolved in dimethylsulfoxide (DMSO; CAS Number 67-68-5; Sigma-Aldrich) and stored in 50 μl aliquots at −80°C. Fresh working solutions were prepared from a thawed BPA stock aliquot for each experiment with the DMSO concentration held constant at 0.1% DMSO, regardless of the BPA concentration being tested.

Oocyte treatment assignment

Sibling GV-stage oocytes were randomly assigned to treatment groups at low magnification (4×) to preclude any possibility of unconscious bias based on morphological appearance. Each oocyte in a sibling pair was cultured either in the presence of a designated BPA concentration dissolved in 0.1% DMSO (Group 1) or in the presence or absence of the solvent to assess any effect of DMSO alone on meiotic maturation (Group 2) as follows: (i) *in vitro* maturation (IVM) medium containing 0.1% DMSO and (ii) IVM medium without DMSO. Allocation to each control was alternated from one patient to the next. When more than two GV-stage oocytes were available from a patient, these 'extra' oocytes were randomly assigned either to an additional BPA concentration for the meiotic stage study, or to one or more of the treatment groups for analyses of spindle integrity and chromosome alignment.

In all, 292 oocytes were assessed for their meiotic status and 175 MII oocytes were examined for spindle integrity and chromosome alignment.

Oocyte fixation and processing for immunofluorescence analysis

Oocytes were fixed at 37°C for 30 min in a microtubule-stabilizing buffer containing 50% deuterium oxide, 2% formaldehyde, 0.1% Triton X-100, 1 μmol/l taxol and 10 IU/ml aprotinin as previously described (Combelles *et al.*, 2002, 2003). Samples were stored at 4°C in a blocking solution containing phosphate-buffered saline (PBS blocking solution) with 0.2% sodium azide, 2% normal donkey serum, 2% bovine serum albumin, 0.1 mol/l glycine and 0.01% Triton X-100.

Oocytes were labeled for tubulin, actin and chromatin for final classification of their maturation status. Fixed oocytes were incubated overnight at 4°C with a mixture of 5 μg/ml monoclonal anti-α-tubulin and anti-β-tubulin primary antibodies (raised against chick and rat brain tubulin, respectively; Sigma-Aldrich). The monoclonal antibodies recognize an epitope at the C-terminal end of their respective tubulin isoforms in a number of organisms, including humans. Samples were washed three times in PBS blocking solution and then exposed for 2 h at 37°C to an Alexa Fluor 488 goat anti-mouse secondary antibody at 2.5 μg/ml (Life Technologies, USA) in PBS blocking solution. Rhodamine-conjugated phalloidin (10 units/ml; Life Technologies) was added to the secondary antibody reagent in order to visualize filamentous actin (microfilaments). Following washing, oocytes were incubated with 15 μg/ml of 4′,6-Diamidino-2-phenylindole (DAPI; Sigma-Aldrich) in PBS blocking solution for 1.5 h at 37°C. Oocytes were mounted in an anti-fading reagent (Vectashield mounting medium; Vector Lab., USA) with minimal compression using a hole reinforcement label.

Classification of oocyte meiotic stages

Labeled oocytes were classified in a blinded fashion by a microscopist not knowing from which treatment group the oocytes were derived (Combelles *et al.*, 2011). Final determination of meiotic stage was obtained after imaging each oocyte using a Zeiss LSM 510 META laser-scanning confocal microscope.

The meiotic stage of each oocyte was defined based on the organization of microtubules, microfilaments and chromatin into normal and abnormal configurations as described below. The use of three labels strengthened the accuracy of meiotic staging; rather than relying only on chromatin or microtubule labeling, microfilament detection allowed the detection of a polar body (PB) with certainty.

Oocytes exhibiting normal meiotic stages

(i) GV (with chromatin patterns and interphase microtubules typical of prophase I oocytes)
(ii) Metaphase I (MI, i.e. with a metaphase-I spindle without a PB)
(iii) Anaphase I (AI, i.e. with a spindle and two groups of segregating chromosomes and without a PB)
(iv) TI (i.e. with a telophase-I spindle and two distinctly separated chromosome clusters, with or without cytoplasmic microtubules)
(v) Pro-metaphase II (PMII, i.e. with an extruded PB, an assembling but not a fully assembled spindle that is typical of metaphase, and condensed chromosomes dispersed within the forming spindle)
(vi) MII (i.e. with a single spindle with chromosomes and an extruded PB).

Oocytes exhibiting abnormal configurations

(i) Oocytes exhibiting GV breakdown (GVBD) with neither an intact GV nor PB, and with aberrant patterns of condensed chromosomes and microtubules.
(ii) Oocytes that exhibited neither a PB nor an intact GV, but that were activated with decondensed chromatin along with the presence of a dense array of cytoplasmic microtubules (Act − PB).
(iii) Oocytes that had extruded a PB and were activated, with decondensed chromatin resembling a pronucleus and a dense array of cytoplasmic microtubules (Act + PB).
(iv) Oocytes that were degenerated (i.e. without detectable microtubules or microfilaments, and having degenerated chromatin).

Representative images of the maturation stages are shown in Fig. 1.

For statistical analyses, oocytes that were at the GVBD stage were grouped with MI and Act − PB oocytes, and those at AI were grouped with those scored as either TI or PMII. Therefore, the proportions of oocytes in a total of six meiotic groups were compared: 1 = GV, 2 = GVBD/MI/Act − PB, 3 = AI/TI/PMII, 4 = MII, 5 = Act + PB; 6 = degenerated.

Analyses of spindle organization and chromosome alignment

Spindle and chromosome organizations in MII oocytes were determined using the Volocity® 3D image analysis software (Perkin-Elmer, Branford, CT, USA). This imaging software allows high-resolution rendering of multichannel 3D data sets in all spindle directions. Spindles were categorized

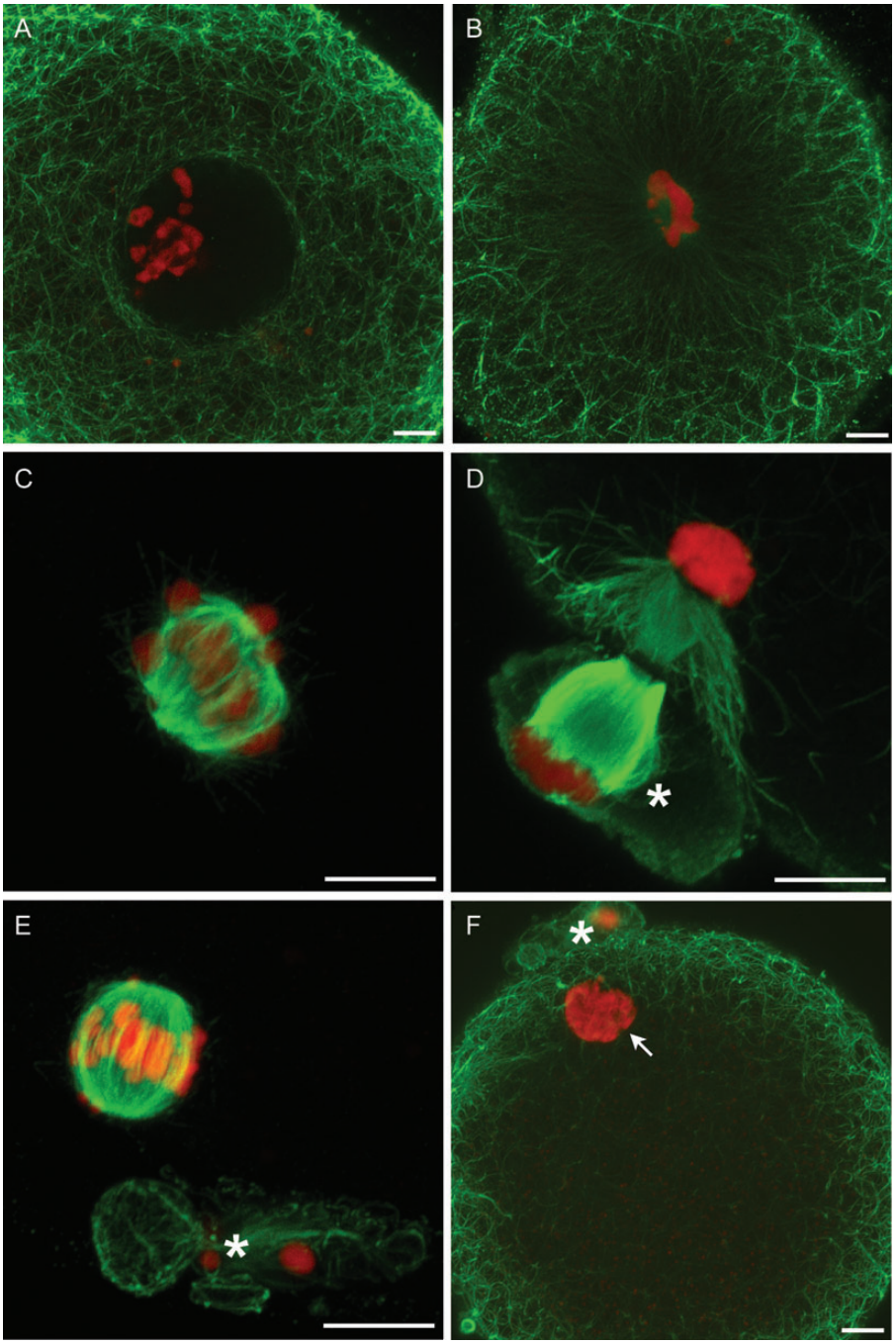

Figure 1 Three-dimensional confocal reconstructions of microtubules (green) and chromatin (red) to show representative meiotic stages of oocytes after culture. (**A**) GV oocyte; (**B**) abnormal GV breakdown; (**C**) metaphase I; (**D**) telophase I; (**E**) metaphase II (MII); (**F**) activated oocyte with a PB. Asterisk indicates the PB. White arrow indicates the pronucleus-like structure. Scale bar: 10 μm.

into one of three groups as previously described by Combelles *et al.* (2011) and Wang *et al.* (2012):

(i) Bipolar spindles (BP), i.e. having two defined and focused poles.
(ii) Bipolar with irregularities (BP*), i.e. having two poles but with irregularities or splayed microtubule fibers at either of the poles or equatorial region.
(iii) Non-bipolar spindles (NBP) that were defined as monopolar, tripolar or any other microtubule structures without apparent organization.

Chromosomes were also classified into one of three groups:

(i) Aligned (A): defined as all chromosomes tightly disposed at the equatorial region of bipolar spindles.
(ii) Slightly dispersed (D*): defined as cases with most chromosomes aligned at an equatorial metaphase plate and 1–6 chromosomes away from the plate.
(iii) Dispersed (D): defined as >6 chromosomes not associated with the metaphase plate.

Representative images of the spindle and chromosome classifications are shown in Fig. 2.

Volumes for all the three spindle categories and pole-to-pole length and equatorial width (for BP and BP* spindles) were obtained from 3D-reconstructions using Volocity®.

Statistical analyses

Our study was designed to detect a 20% decrease in the percentage of GV-stage oocytes maturing to MII in the 20 ng/ml BPA concentration group when compared with the pooled control group, at 80% power and alpha = 0.05 (e.g. from 75% progression to MII in the control group to 55% in the 20 ng/ml BPA group). Under these assumptions, we calculated that we would need 88 oocytes in each group to detect a statistically significant difference in progression to MII.

McNemar's test for marginal homogeneity using the exact binomial distribution (a comparison of discordant pairs) was used to compare the dichotomized maturation status (e.g. MII versus non-MII) when comparing two exposure groups (i.e. control versus BPA).

Results from the two experiments were pooled to assess whether there was a dose–response association between oocyte stage (when compared with all other stages). Tests for trend were performed by including log BPA as an ordinal predictor; the log of 0.00001 was substituted for the control group. Generalized estimating equations (GEEs) with a binomial distribution, log link and compound symmetry covariance structure were used to account for the correlation between oocytes from the same woman. GEE models with robust standard errors were also used in the spindle analysis to account for multiple oocytes from the same woman. A binomial distribution and logit link were applied for dichotomous outcomes (spindle morphology), and the normal distribution and identity link were applied for continuous outcomes (length, width, volume). All analyses were performed using Statistical Analysis Software (SAS®) version 9.2 (SAS Institute, Inc., Cary, NC, USA). A P value of <0.05 was considered significant.

Results

Patients and oocytes

A total of 352 GV-stage oocytes from 121 patients were released from the clinical IVF laboratory for use in this study. Of these, 292 were randomly assigned to the meiotic stage experiments (71 patients contributed 2 oocytes, 50 patients contributed 3 oocytes). The remaining 60 oocytes were used to increase the sample size of MII oocytes available for the spindle/chromosome alignment analyses. The demographic characteristics of the 121 patients from whom oocytes were obtained are shown in Table I. The study included pairs of immature oocytes from seven donors. Only in one pair of donor oocytes, did both oocytes (control and BPA 20 ng/ml) mature to MII.

Maturation in the control groups

No difference was observed between the proportion of oocytes that progressed to MII after culture in medium with or without 0.1% DMSO [62% (38/61) versus 55% (33/60), respectively]. Therefore, data from the two control groups were pooled for further analyses (referred to henceforth as the 'control').

BPA exposure and incidence of MII oocytes

Oocytes exposed to 20 ng/ml BPA progressed to MII with a similar incidence compared with controls [52% (52/100) versus 55% (55/100), respectively; $P = 0.74$; 100 sibling pairs from 100 patients]. Oocytes exposed to 200 ng/ml BPA progressed to MII at a lower incidence compared with controls but this difference was of borderline significance [42% (21/50) versus 58% (29/50), respectively; $P = 0.06$; 50 sibling pairs from 50 patients]. In contrast, oocytes exposed to 20 μg/ml BPA progressed to MII at a significantly lower incidence compared with control oocytes [19.0% (4/21) versus 76.2% (16/21), respectively, $P = 0.002$; $n = 21$ sibling pairs from 21 patients].

BPA exposure and incidence of non-MII oocytes

Analysis of those oocytes not classified at MII revealed no differences between the BPA exposed and corresponding sibling controls for any stage with the exception of Act + PB oocytes after exposure to 200 ng/ml BPA. For these oocytes, there was a significantly increased incidence of Act + PB compared with the control group [20% (10/50) versus 4% (2/50), respectively; $P = 0.04$]. Table II shows the distribution of oocytes by meiotic classification after exposure to each of the three concentrations of BPA tested.

BPA dose–response and oocyte maturation

In order to determine whether a dose–response relationship existed between BPA and oocyte maturation status, the results across the three concentrations of BPA tested were analyzed using the GEE approach. Control oocytes from both experiments were also combined in one group ($n = 121$) and the percentage of oocytes at each maturation stage was re-assessed. As BPA concentration increased, there was a significant decrease in the proportion of oocytes reaching MII compared with those not at MII ($P = 0.002$), and a significant increase in the proportion of Act + PB oocytes compared with other maturation stages ($P = 0.007$). As BPA concentration increased there was also a significant increase in the proportion of oocytes that were degenerated compared with those that were not degenerated ($P = 0.01$). Of the oocytes classified as degenerated (control oocyte and BPA-exposed oocytes, pooled) ($n = 20$), 7 had degenerated at the GV-stage, 11 had undergone degeneration at the GVBD stage, and the remaining 2 had degenerated at MI.

Spindle formation and chromosome alignment in MII oocytes

One hundred and seventy five MII oocytes were evaluated for spindle organization and chromosomal alignment (Fig. 2; control: $n = 80$, 20 ng/ml BPA: $n = 63$, 200 ng/ml BPA: $n = 27$, BPA 20 μg/ml: $n = 5$). Patient age, BMI and infertility diagnoses were comparable among the groups (Supplementary data, Table SI). There was a significant negative dose–response association of BPA with BP in the oocytes (Table III) as well as aligned chromosomes (Table IV). Combining the incidence of oocytes with BP (Fig. 2A) and BP* spindles (i.e. spindles with irregular two poles) (Fig. 2D), we found no difference among the BPA groups. There was no difference in NBP spindles (NBP; Fig. 2E) among the BPA groups. As the concentration of BPA increased, the percentage of BP with focused poles and aligned chromosomes decreased. Exposure to 20 ng/ml of BPA was associated with a significant decrease in the incidence of bipolar, focused spindles [odds ratio (OR) = 0.22, confidence interval (CI) = 0.11–0.44; two-sided Wald P-value < 0.0001] compared with controls, and was associated with a significantly lower likelihood of aligned chromosomes compared with controls [OR = 0.51, CI = 0.28–0.94; two-sided Wald P-value = 0.03]. There was no

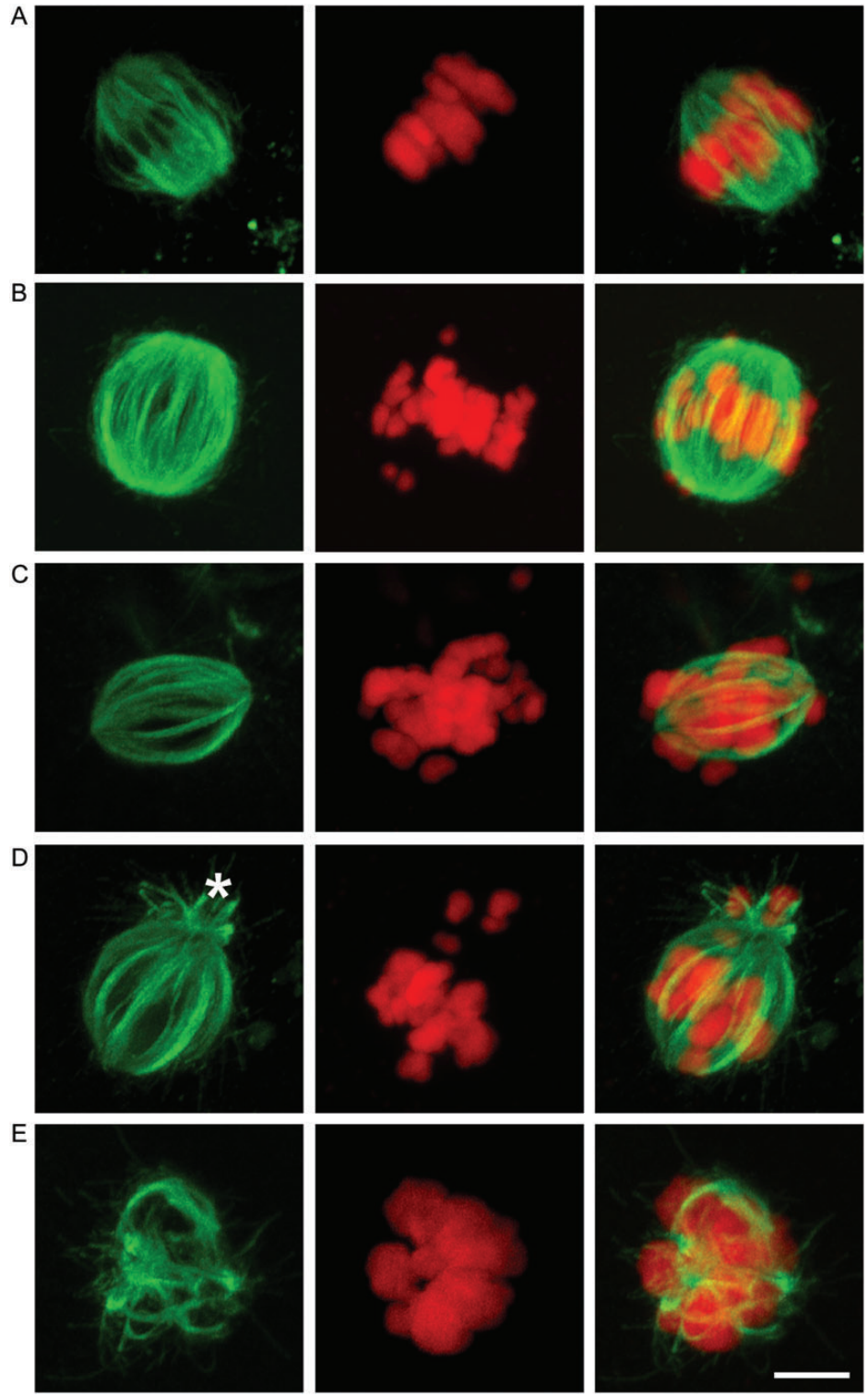

Figure 2 Representative classifications of spindle morphology and chromosome alignment in MII oocytes, with microtubules in green (left panels), chromosomes in red (middle panels) and merged images (right panels). Examples include: (**A**) bipolar (BP) spindle with aligned chromosomes; (**B**) BP spindle and some chromosomal dispersal, with most chromosomes aligned with the exception of three chromosomes displaced from the spindle equator; (**C**) BP spindle with chromosomes dispersed throughout the spindle; (**D**) BP spindle with irregularities (*), notably at one of the spindle poles (asterisk), and some chromosomal dispersal (D*); (**E**) Non-bipolar spindles (NBP) with chromosomes dispersed (D) within the spindle structure. Scale bar: 5 μm.

significant difference between the proportion of focused BP spindles between controls and oocytes exposed to 200 ng/ml BPA [OR = 0.50, CI = 0.20–1.27; two-sided Wald *P*-value = 0.15) or the likelihood of aligned chromosomes compared with controls [OR = 0.76, CI = 0.32–1.79; two-sided Wald *P*-value = 0.53). However, none of the oocytes exposed to the 20 μg/ml BPA had a BP spindle and all of these NBP spindles showed dispersed chromosomes.

Although the sample size was not sufficiently large to study BPA effects on spindle morphology and chromosome alignment in sibling MII oocytes, we did look descriptively at a stratified analysis for the 16 pairs in which both the control and BPA exposed oocytes (20 ng/ml) were classified as MII. As shown in Supplementary data, Tables S2 and S3, respectively, the relationships between BPA exposure and spindle morphology as well as BPA exposure and chromosome alignment were consistent with those observed in the overall population of oocytes examined. Spindle morphology and chromosome alignment were altered in a similar way in sibling oocyte pairs as in the pooled population.

Evaluation of the length and width of the BP spindles revealed no difference between controls and 20 ng/ml BPA (Table V). However, when

Table I Characteristics at start of IVF cycle of 121 patients from whom oocytes were retrieved.

Characteristic	All patients (*n* = 121)
Age (years)	
Mean ± SD	35.5 ± 4.9
Range	23.9–43.9
BMI kg/m^2	
Mean ± SD	25.1 ± 5.5
Range	17.8–42.0
Infertility diagnosis	
Male factor only	61 (50.4%)
Male + female factor	53 (43.8%)
Donor oocytes	7 (5.8%)
Stimulation protocol	
LDLL	76 (62.8%)
Antagonist	19 (15.7%)
Poor responder	26 (21.5%)
Race	
White	80 (66.1%)
Asian	18 (14.9%)
Hispanic	5 (4.1%)
Black	8 (6.6%)
Other	3 (2.5%)
Not reported	7 (5.8%)

LDLL, low-dose luteal lupron.

Table III Prevalence of Bipolar Spindles in MII oocytes according to BPA exposure.

BPA group	No. oocytes	No. oocytes with a BP (%)	OR (95% CI)[a]	*P*-value[a]
Control	80	57 (71.3%)	1.00 (reference)	Reference
BPA, 20 ng/ml	63	22 (34.9%)	0.22 (0.11–0.44)	<0.0001
BPA, 200 ng/ml	27	15 (55.6%)	0.50 (0.20–1.27)	0.15
BPA, 20 μg/ml	5	0 (0.0%)	–[b]	–
Test for trend[c]				<0.0001

[a]OR, 95% CI and *P*-values were derived from GEEs with robust standard errors to account for the correlation between oocytes from the same woman. A binomial distribution and logit link were applied.
[b]This group was excluded from analysis due to the small sample size.
[c]The test for trend *P*-value was obtained from a model that includes log BPA as an ordinal predictor. The log of 0.00001 was substituted for the Control group.

Table II The association of BPA and the progression of human oocyte maturation among 100 sibling oocytes exposed to 20 ng/ml BPA, 50 sibling oocytes exposed to 200 ng/ml and 21 sibling pairs exposed to 20 μg/ml BPA.

Meiotic stage	20 ng/ml BPA (*n* = 100 sibling pairs)[a]		200 ng/ml BPA (*n* = 50 sibling pairs)		20 μg/ml BPA (*n* = 21 sibling pairs)	
	Control	BPA	Control	BPA	Control	BPA
GV	13 (13.0)	10 (10.0)	6 (12.0)	3 (6.0)	1 (4.8)	6 (28.6)
GVBD/Act − PB/MI	16 (16.0)	12 (12.0)	9 (18.0)	8 (16.0)	0 (0)	4 (19.1)
AI/TI/PMII	8 (8.0)	5 (5.0)	3 (6.0)	3 (6.0)	3 (14.3)	0 (0)
MII	55 (55.0)	52 (52.0)	29 (58.0)	21 (42.0)	16 (76.2)	4 (19.1)[b]
Act + PB	5 (5.0)	12 (12.0)	2 (4.0)	10 (20.0)[c]	1 (4.8)	4 (19.1)
Degenerated	3 (3.0)	9 (9.0)	1[d] (2.0)	5 (10.0)	0 (0)	3 (14.3)

Numbers in parentheses are percentages of the total number of oocytes cultured in that treatment group. GV, germinal vesicle; GVBD, germinal vesicle breakdown; Act − BP, activated polar body; MI, metaphase I oocytes; AI, anaphase I oocytes; TI, telophase I oocytes; MII, metaphase II oocytes; Act + PB, activated + polar body.
[a]Of the 100 women providing the 100 sibling pairs used to test 20 ng/ml BPA, 50 women contributed a third oocyte which was cultured with 200 ng/ml BPA.
[b]Significantly fewer MII oocytes versus non-MII oocytes after exposure to 20 μg/ml BPA compared with the corresponding control; *P* = 0.002.
[c]Significantly more Act + PB oocytes versus non-Act + PB oocytes after exposure to 200 ng/ml BPA compared with the corresponding control; *P* = 0.04 (McNemar's test for marginal homogeneity).
[d]This control oocyte was also included as one of the controls for BPA 20 ng/ml as it was one of three sibling oocytes.

Table IV Chromosome alignment according to BPA exposure in MII oocytes.

BPA group	No. oocytes	No. oocytes with aligned chromosomes (%)	OR (95% CI)[a]	*P*-value[a]
Control	80	41 (51.3%)	1.00 (reference)	Reference
BPA, 20 ng/ml	63	22 (34.9%)	0.51 (0.28–0.94)	0.03
BPA, 200 ng/ml	27	12 (44.4%)	0.76 (0.32–1.79)	0.53
BPA, 20 μg/ml	5	0 (0.0%)	–[b]	–
Test for trend[c]				0.02

[a]ORs, 95% CIs and *P*-values were derived from GEEs with robust standard errors to account for the correlation between oocytes from the same woman. A binomial distribution and logit link were applied.
[b]This group was excluded from analysis due to the small sample size.
[c]The test for trend *P*-value was obtained from a model that includes log BPA as an ordinal predictor. The log of 0.00001 was taken for the control group.

Table V Spindle measurements according to BPA exposure in MII oocytes.

BPA group	Length[a]	Width[a]	Volume, BPs	Volume, NBPs
Control				
n	72	72	71	7
Mean ± SD	10.6 ± 1.9 (reference)	8.8 ± 1.1 (reference)	428.6 ± 134.4 (reference)	489.7 ± 183.6 (reference)
BPA, 20 ng/ml				
n	57	57	57	5
Mean ± SD	10.5 ± 1.7	9.1 ± 1.1	431.5 ± 145.1	402.9 ± 89.0
Δ in Mean[b] (*P*-value[c])	−0.10 (0.77)	0.26 (0.15)	2.8 (0.90)	−86.7 (0.24)
BPA, 200 ng/ml				
n	24	24	24	3
Mean ± SD	11.1 ± 1.9	9.5 ± 1.0	529.0 ± 197.6	569.6 ± 211.6
Δ in Mean[b] (*P*-value[c])	0.50 (0.30)	0.63 (0.008)	100.3 (0.02)	79.9 (0.41)
BPA, 20 μg/ml				
n	—	—	—	4
Mean ± SD				98.3 ± 57.2
Δ in Mean[b] (*P*-value[c])				−391.3 (<0.0001)

BP, bipolar; NBP, non-bipolar.
[a]Calculated for BPs only.
[b]Difference in the mean compared with the corresponding reference.
[c]*P*-values are from a GEE model, with a normal distribution and identity link.

compared with oocytes in the control group, oocytes exposed to 200 ng/ml BPA had, on average, a larger mean volume of the BP (100.3 μm^3 higher, $P = 0.02$) as well as, on average, wider spindles ($P = 0.008$). Oocytes exposed to 20 μg/ml BPA had significantly smaller NBP, with a mean difference of −391 μm^3 compared with controls ($P < 0.0001$). There were no differences in either the width or length of BP* spindles among the BPA groups.

Discussion

To our knowledge, this is the first study that has investigated the association between BPA exposure and maturation of human oocytes *in vitro*. Our observations indicate that BPA is associated with interferences in the progression of meiotic maturation as well as disruption of spindle organization and chromosome alignment. Increased BPA exposure was associated with a decreased percentage of oocytes progressing to MII, and an increased percentage of oocytes undergoing activation or becoming degenerated. A significant dose–response effect of BPA on spindle bipolarity and chromosome alignment was also observed. Since sibling oocytes were used in this study, these findings were independent of the effect of potential confounders from patient heterogeneity such as patient age at cycle start, BMI, infertility diagnosis, race, stimulation protocol and any prior exposure of oocytes to BPA *in vivo*.

Studies of this type with human oocytes are challenged by the paucity and heterogeneity of material available. Indeed, consistent with such expected heterogeneity, we did see variation regarding the proportion of control oocytes that matured to MII in the first experiment (16/21,

76.2%) when compared with that in the second experiment (55/100, 55%). This variation may have been due to the small sample size in the first experiment ($n = 21$) as well as to biological variability. However, this variation likely did not confound our findings as our experimental design used sibling oocytes within each patient with one oocyte always cultured in control conditions and another exposed to a BPA condition. It is relevant to note that we used more stringent criteria for classifying oocytes at MII when compared with most previous reports that relied on the detection of an emitted PB by light microscopy. We used confocal microscopy analyses of spindles, microfilaments and chromosomes to distinguish +PB oocytes that were true MII oocytes from those that were either T-I or Act + PB. The relatively low incidence of MII we observed for all oocytes cultured in control conditions (71/121; 58.7%) contrasts the ~75% rate reported in two other studies using the same IVM medium after culture for 36 h (Combelles *et al.*, 2011; Wang *et al.*, 2012). However, this disparity in maturation rates may be explained by our shorter culture time of 30 h in the present study.

Despite failing to observe a significant effect of 20 ng/ml BPA on progression to MII when compared with corresponding control oocytes, the effect of 200 ng/ml BPA on the progression to MII was almost statistically significant ($P = 0.06$), despite the small sample size ($n = 50$). Overall, a significant dose-dependent relationship was revealed when we combined the data among the three BPA concentrations and used GEE statistical methods for data analysis (Table II). We also found a significant dose–response effect of BPA on the incidence of MII oocytes with BP and aligned chromosomes. All the oocytes exposed to 20 μg/ml BPA had NBP and dispersed chromosomes. These results require further investigation since the sample size was very small ($n = 5$) due to the low maturation rate in this group. Of interest, exposure to 20 ng/ml BPA resulted in a significant decrease in the prevalence of BP (compared with controls) but no statistical difference was observed after exposure of oocytes to 200 ng/ml (Table III). In fact, the percentage of BP in the 200 ng/ml group was higher than that in the 20 ng/ml group (55.6% versus 34.9%, respectively, NS). Although these results might be attributed to the small sample size of oocytes studied in these groups, they may be related to a non-linear response to BPA doses (Jones *et al.*, 2011; Klingmüller and Allera, 2011; Vandenberg *et al.*, 2012). Further work is required to investigate this possibility.

Taken together, these observations are consistent with numerous animal studies demonstrating that BPA exposure causes meiotic arrest of oocytes and spindle abnormalities both *in vivo* and *in vitro*. Hunt *et al.* (2003) were the first to incidentally observe meiotic disturbances among mice that were housed in damaged cages that resulted in leaching of BPA. In a subsequent study, these investigators administered BPA for 6–8 days in doses between 20 and 100 ng/g body weight to pre-pubertal mouse pups. They showed that *in vivo* BPA exposure during the final stages of oocyte maturation was correlated with meiotic abnormalities and increased rates of chromosome congression failure.

In vitro studies in mice involving either cumulus–oocyte complexes or denuded oocytes have shown that short exposure to BPA during the final stages of oocyte maturation is associated with cell cycle delays and spindle abnormalities (Can *et al.*, 2005; Eichenlaub-Ritter *et al.*, 2008; reviewed by Hunt *et al.*, 2009). Indeed, Can *et al.* (2005) exposed mice cumulus–oocyte complexes to two different concentrations of BPA [10 μM (2.28 μg/ml) and 30 μM (6.84 μg/ml)] (for up to 18 h) and compared the maturation status of the oocytes to controls. Similar to our findings, *in vitro* exposure to BPA caused a dose-dependent inhibition of cell cycle progression with a decreased number of oocytes progressing to MII as well as an increased incidence of spindle abnormalities.

Depending on the BPA dose, oocytes showed spindle abnormalities, such as loosening and elongation of meiotic spindles and compaction/dispersion of pericentriolar material. Mouse oocytes exposed to 10 μM BPA exhibited significantly altered spindles, with spindle elongation as the most common abnormality. Similar to our findings, Can *et al.* (2005) reported a rise in the incidence of small MII spindles after exposure to high concentrations of BPA (30 μM). Exposure to 10 and 30 μM BPA resulted in very few or even no chromosomes in the PB, perhaps indicating that BPA may interfere with chromosome segregation and lead to the formation of hyperploidic MII oocytes. Eichenlaub-Ritter *et al.* (2008) exposed denuded mouse oocytes *in vitro* (for 16 h) to a wide range of concentrations of BPA (50, 100, 200, 400, 800 ng/ml and 4, 10 μg/ml). Only oocytes cultured in medium containing 10 μg/ml BPA exhibited a significantly higher incidence of meiotic arrest and spindle abnormalities (unfocused and broad poles), with no increase in BPA-related aneuploidy. Prior reports have not analyzed spindle dimensions, and the biological relevance of our reported increase in spindle width in human oocytes exposed to 200 ng/ml of BPA remains to be determined. Our findings, together with prior animal studies, support the negative influences of BPA on cell cycle progression, spindle architecture and chromosome organization during oocyte maturation.

Unexpectedly, we identified a BPA dose–response increase in the incidence of activated oocytes with an extruded PB. It is not known whether BPA exposure is associated with an increased instability at MII resulting in spontaneous activation, or whether BPA induces activation directly after TI, without the oocytes reaching MII. Further studies using time-lapse microscopy are required to distinguish between these possibilities. Whether BPA may directly affect the regulation of the female meiotic cell cycle also merits investigation, notably in light of our findings and the ability of BPA to modulate the expression of cell cycle factors in cultured mouse antral follicles (Peretz *et al.*, 2012).

We also observed a dose–response effect of BPA exposure on the proportion of degenerated oocytes. As BPA concentration increased, more oocytes degenerated, with the majority degenerating during GVBD. Consistent with this finding, Brieño-Enríquez *et al.* (2011) documented a significantly increased incidence of degeneration in human oocytes exposed to BPA (1–30 μM, i.e. 230 ng/ml to 6.9 μg/ml), albeit the exposure was for longer durations (7–21 days). Increased rates of degenerated oocytes associated with BPA exposure may be relevant to our understanding of the decrease in fertility reported in the last decades (Guzick and Swan, 2006; Hamilton and Ventura, 2006). Indeed, it is possible that chronic exposures to BPA and other EDCs during the reproductive lifespan are associated with higher rates of oocyte degeneration. Previous animal studies have not documented BPA-associated increases in either the incidence of degeneration or spontaneous activation (Hunt *et al.*, 2003; Can *et al.*, 2005; Eichenlaub-Ritter *et al.*, 2008) and it may be that human oocytes are particularly susceptible to such developmental aberrations. However, one must consider whether oocytes that are developmentally compromised may respond to BPA differently from 'normal' oocytes; of note, the oocytes used in the Brieño-Enríquez *et al.* (2011) study and in the experiments reported here may not represent developmentally competent oocytes; they were, respectively, from fetal ovaries and the pool of oocytes that failed to mature *in vivo*.

The mechanism of action of BPA on oocyte maturation is currently unknown. Since BPA is an estrogenic compound, it is interesting to

note that E_2 impairs meiotic progression and spindle organization in bovine oocytes during *in vitro* maturation (Beker-van Woudenberg *et al.*, 2004). During folliculogenesis, BPA disrupts cell cycle progression and induces atresia. Peretz *et al.* (2012) showed that in a mouse model, BPA disrupts the normal cell cycle by significantly increasing Cdk4, Ccne1 and Trp53 expression and significantly decreasing Ccnd2 expression in the follicle, as well as inducing follicular atresia and increased expression of Bax. However, these effects were shown only after exposure to 100 μg/ml BPA, which are very high and not physiological levels. Another study by Dong *et al.* (2011) has suggested that BPA acts through non-genomic estrogen pathways (GPR30 and ERK pathways) in breast cancer cells. Taken together, current studies indicate that the mechanism of action of BPA on oocyte maturation merits further investigation, including the receptor pathway by which BPA is acting in the oocyte.

The present investigation has several limitations. First, given that we had access only to clinically discarded oocytes, most of them from infertile patients, we were limited to evaluating only those oocytes that failed to mature *in vivo* despite having been exposed to gonadotrophin stimulation and the ovulatory trigger of HCG. Although starting with such potentially compromised material might reduce the relevance of our findings to a more normal population of oocytes, our sibling oocyte experimental design and the fact that the majority of GV oocytes matured under control conditions, support utility of this model for this study. Of interest is the fact that the study included seven pairs of donor oocytes. Although such oocytes likely represent those from the normal fertile population, they responded to BPA in a manner similar to those from the infertile population. The second limitation is the duration of oocyte exposure to BPA. The oocytes were exposed to BPA only during the period for meiotic maturation *in vitro*, thus not taking into consideration any possible long-term effects of exposure during folliculogenesis *in vivo*. As BPA has been detected in the follicular fluid of IVF patients (Hauser *et al.*, unpublished data), such chronic exposure to BPA, as well as other EDCs, may impact oocyte quality at low concentrations. The present study showed a slight, albeit insignificant decrease in maturation rates following exposure to the lower doses of 20 and 200 ng/ml BPA compared with controls. Therefore, the possibility exists that enlarging the sample size may reveal significant differences among the groups even at the lowest BPA concentration tested in this study (Hauser *et al.*, unpublished data). A recent study by Ehrlich *et al.* (2012a,b) did show an association between urinary BPA levels and ovarian response among IVF patients. The authors reported a significant decrease in the average number of MII oocytes and number of normally fertilizing oocytes in relation to urinary BPA concentrations (dose–response association). These clinical findings are similar to our *in vitro* results using compromised human material.

In summary, our preliminary observations document impairment of human oocyte maturation by BPA *in vitro* and provide novel insight into a possible effect of BPA on oocyte maturation *in vivo*. The observed dose–response effects of BPA on progression to MII, the prevalence of bipolar spindles with focused poles and aligned chromosomes, and the incidence of spontaneous activation and degeneration suggest that BPA may be associated with compromised meiotic competency of oocytes following the pre-ovulatory gonadotrophin surge *in vivo*. Further work is warranted to investigate this possibility, to test the effect of lower BPA concentrations (1–10 ng/ml) and to identify the mechanism(s) underlying the BPA-associated perturbations in oocyte maturation.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

R.M. performed the culture, fixation and labeling of oocytes as well as the immunofluorescence scoping and writing the manuscript. C.C. participated in the study design, performed the immunofluorescence and confocal imaging and edited the manuscript. S.M. participated in the study design, conducted the data analyses and edited the manuscript. K.C. conducted the data analysis and edited the manuscript. P.W. participated in the study design and data analysis and edited the manuscript. R.H. participated in the experimental design, editing and finalizing of the manuscript. C.R. was responsible for the experimental design, overseeing completion of the study, editing and finalizing of the manuscript.

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Conflict of interest

The authors can identify no potential conflicts of interest, neither financial nor any other, involved in the writing or publication of this manuscript.

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