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Bisphenol-A induces cell cycle delay and alters centrosome and spindle microtubular organization in oocytes during meiosis

A.Can[1,2,4], O.Semiz[3] and O.Cinar[1]

[1]Laboratory for Reproductive Cell Science, Department of Histology–Embryology, Ankara University School of Medicine, Ankara, 06100, [2]Ankara University Biotechnology Institute, Besevler, Ankara, 06500 and [3]Sakarya University School of Health, Esentepe Campus, Sakarya 54187, Turkey

[4]To whom correspondence should be addressed at: Department of Histology–Embryology, Ankara University School of Medicine, Ankara, Sihhiye 06100, Turkey. E-mail: alpcan@medicine.ankara.edu.tr

Bisphenol-A (BPA) is a widely used environmental estrogen-like chemical that has a weak estrogenic activity. This study aimed to test the potential inhibitory effects of BPA on meiotic cell cycle progression, centrosomes and spindle integrity in mouse cumulus–oocyte complexes (COCs). They were exposed to BPA (10–30 μM; 2.3–6.8 ppm) during meiosis-I and the formation of metaphase-II (M-II) spindle. Exposure to BPA during meiosis-I caused a dose-dependent retardation/inhibition of cell cycle progression; 74 and 61% of cells reached metaphase-I (M-I) in the presence of 10 and 30 μM BPA, respectively, (81% in controls, $P < 0.001$). A more striking delay was noted when oocytes were exposed to BPA during the formation of M-II spindle, i.e. 61 and 41% of cells (94% in controls, $P < 0.001$) reached M-II while the remaining cells remained at M-I. Depending on dose, both (i) loosening and elongation of meiotic spindles and (ii) compaction and dispersion of pericentriolar material (PCM) were noted in all samples, all of which resulted in a series of spindle abnormalities. Interestingly, no chromosome was detected in the first polar body after the 10 and 30 μM BPA treatments. When the cells were freed from BPA exposure at 10 and 30 μM, 70 and 61%, of the cells succeeded in reaching M-II (93% in controls, $P < 0.001$), respectively. In conclusion, one mode of action of BPA is a moderately severe yet reversible delay in the meiotic cell cycle, possibly by a mechanism that degrades centrosomal proteins and thus perturbs the spindle microtubule organization and chromosome segregation.

Key words: bisphenol-A/centrosome abnormality/meiotic spindle/oocyte/pericentrin

Introduction

Bisphenol-A (BPA), an alkylphenol, is an example of an industrial estrogen-like chemical that has a weak estrogenic activity (1000-fold lower than observed for 17β-estradiol) (Deodutta *et al.*, 1997). It is used in the manufacture of epoxy, polycarbonate and corrosion-resistant polyester-styrene resins required for food packaging materials in industrial processing (Gilbert *et al.*, 1982). BPA have been shown to leach from plastic used in food processing and wrapping (NIOSH, 1985; Krishnan *et al.*, 1993), from lacquer coating in food cans (Brotons *et al.*, 1995) ranging from 0 to 33 μg per can. BPA is also used as a dental sealant and has been reported to be found in the saliva of patients having dental sealants (Fung *et al.*, 2000).

Estrogen-like chemicals are unique compared to non-estrogenic xenobiotics, because in addition to their chemical properties, the estrogenic property of these compounds allows them to act like sex hormones. Most phytoestrogens and synthetic compounds including BPA bind to estrogen receptor ERα and ERβ with relatively low affinity (Kuiper *et al.*, 1997). The synthetic estrogens are capable of inducing distinct patterns of ER agonist/antagonist activities that are cell context- and promoter-dependent, suggesting that these compounds will induce tissue-specific *in vivo* ER agonist or antagonist activities.

Humans are exposed to estrogenic compounds present in vegetables, fruits and meat on a regular basis (Li *et al.*, 1985; Markaverich *et al.*, 1995). While physiological concentrations of estrogen are essential for the maintenance of cell growth such as cell proliferation, transcription and DNA synthesis (Jensen, 1992), an imbalance in the steady-state concentrations, pharmacological dose or concentration higher than the physiological level of estrogens is known to produce adverse effects (Marselos and Tomatis, 1992). Therefore, estrogen-like chemicals that are widely available in human micro- and macro environments are potential agents that interfere with endogenous estrogen concentrations.

The exposure of both female and male rats and mice to BPA has been reported to produce adverse reproductive effects such as increased prostate size, decreased epididymal weight, increased androgen receptor binding activity (Nagel *et al.*, 1997), inducement of an uterotrophic response, reduced estrous cycle (Kim *et al.*, 2001; Laws *et al.*, 2000). Dose and duration experiments on cell-free systems and cultured somatic cells or embryos have been evaluated to test the cellular toxicity of BPA, especially its reproductive toxicity. Takai *et al.* (2000) found that low doses of BPA (1–3 nM) promote the development of 2-cell mouse embryos, while 100 μM concentrations inhibit developmental progression. Similarly, Kim *et al.* (2001) reported that 1–10 μM BPA induced cell proliferation.

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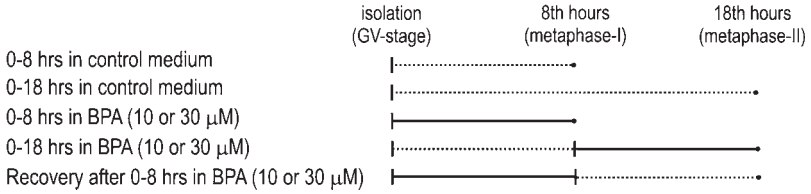

Figure 1. The experimental design for BPA treatment of cultured mouse COCs. Solid lines indicate periods of drug exposure and dotted lines refer to culture periods in control medium. Round black dots indicate the time points of removing the cumulus cells and fixation of oocytes.

Pfeiffer *et al.* (1997) studied the aneuploidogenic potential of BPA in cell-free systems and in cell cultures and they demonstrated that BPA inhibits the polymerization and increases the depolymerization of microtubules. V79 cell lines were exposed to various endocrine-disrupting agents, including BPA, by Nakagomi *et al.* (2001), and results showed that BPA displayed a moderate to severe microtubule-disrupting effect at 75 μM doses, among other effects. More recently, Hunt *et al.* (2003) reported that BPA induces aneuploidy in maturing oocytes. Despite increasing data about BPA regarding its aneuploidogenic effects, no direct study has previously focused on BPA-related adverse effects regarding meiotic microtubules and meiotic cell cycle machinery, which play major roles in proper chromosome movement during meiosis.

We have previously shown the inhibitory effects of diethylstilbestrol (DES), a structurally similar compound to BPA, on mouse meiotic cell division machinery and concluded that DES mainly interferes with centrosomes and microtubule dynamics, resulting in a dramatic block of cell division during critical stages of development in meiotically competent oocytes (Can and Semiz, 2000). Given the similar but smaller effects of BPA in this study, we tested potential perturbations in microtubular and centrosomal function in isolated mouse oocytes subsequent to an *in vitro* exposure of low doses of BPA (10–30 μM) during meiosis-I through to the formation of the M-II spindle. Results showed that BPA causes a time- and dose-dependent delay in cell cycle progression by interfering mainly with centrosomal proteins, and to a lesser extent with microtubules, which ultimately leads to the formation of abnormal spindle and chromosome non-disjunction.

Materials and methods

Collection, culture and fixation of oocytes

Cumulus–oocyte complexes (COCs) ($n = 1561$) were obtained from 19–21-day old Balb/c mice injected 48 h earlier with 5 IU of equine chorionic gonadotrophin (Sigma Co., USA). COCs were transferred into a collection medium (Eagle's MEM with Hanks salts and buffered with HEPES (pH = 7.3), supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.3% bovine serum albumin (BSA) and then incubated in maturation medium (Eagle's MEM supplemented with Earle's salts, 2 mM glutamine, 0.23 mM pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.3% BSA) in the presence (see below) or absence of BPA. After removal of cumulus cells by gentle pipetting, denuded oocytes were fixed/extracted for 20 min at 37°C in a microtubule-stabilizing buffer containing 2% formaldehyde, 0.5% Triton-X 100, 1 μM taxol, 10 U/ml aprotinin and 50% deuterium oxide. The samples were washed three times in a blocking solution of phosphate-buffered saline (PBS) containing 2% BSA, 2% powdered milk, 2% normal goat serum, 0.1 M glycine and 0.01% Triton X-100 and stored at 4°C in blocking solution until processing.

BPA treatment of oocytes during in vitro *maturation*

BPA (4,4′-isopropylidenediphenol; MW: 228.3; purity = 95%; CAS Number: 80-05-7) (Sigma-Aldrich, USA) was dissolved in dimethylsulphoxide (DMSO) as a 10 mM stock solution and freshly prepared working solutions of 10 and 30 μM in oocyte maturation medium were used to treat COCs during different stages of meiotic division. The DMSO concentration of treated and control samples never exceeded 0.1% (v/v) in working solution, and at a given concentration, no adverse effects of DMSO on oocyte maturation were observed.

COCs were treated during initial stages of *in vitro* maturation for 8 h between germinal vesicle (GV)-stage and M-I at 10 μM ($n = 225$) and 30 μM ($n = 232$) (see Figure 1 for experimental design). A different set of COCs was exposed to BPA ($n = 261$ at 10 μM; $n = 240$ at 30 μM) during an 8–18 h-interval between M-I and M-II. To test the reversibility of the effects appearing during this stage, a group of COCs ($n = 283$) were washed free of the drug, and then cultured for an additional 10 h in control medium. The control group ($n = 320$) was cultured for 8 or 18 h in control medium.

Fluorescent labelling of oocytes

A triple fluorescent staining procedure was performed using antibodies raised against α + β-tubulin and pericentrin (Can *et al.*, 2003a)–a major centrosomal protein located in pericentriolar material (PCM), which is responsible for the nucleation and assembly of meiotic spindles during meiosis-I and II (Doxsey *et al.*, 1994). Oocytes were incubated with primary and secondary antibodies for 2–5 h per antibody at either 4 or 37°C in a rotating shaker followed by 15 min washes in a PBS-blocking solution between incubation steps. Microtubules were visualized using a 1:1 mixture of anti-α + β tubulin mouse monoclonal antibodies (1:100 dilution) (Sigma-Aldrich, USA), followed by a 1:100 dilution of an affinity-purified fluorescein isothiocyanate (FITC) goat anti-rat IgG (Jackson ImmunoResearch Laboratories, PA, USA). Centrosomes were labelled with a rabbit polyclonal antibody directed against pericentrin (4B) (Doxsey *et al.*, 1994) at a final dilution of 1:100, followed by a 1:100 dilution of an affinity-purified Cy5-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, PA, USA). For the evaluation of chromatin and chromosomes at specific stages in meiosis, oocytes were dual-stained with 10 mM of 7-aminoactinomycin-D (7-AA-D) (Sigma-Aldrich, USA) (Can *et al.*, 2003b) and Hoechst 33258 (Polyscience Inc, PA, USA). Finally, oocytes were mounted between glass coverslips and slides using spacers, allowing a ~100 μm space in between (Can, 1996), which was filled with a 1:1 glycerol/PBS medium containing 25 mg/ml sodium azide as an anti-fading reagent.

3D Image reconstruction by confocal laser scanning microscopy

Labelled oocytes were initially examined by a Zeiss Axiovert 100 M inverted microscope using conventional UV filter set and mercury-arc lamp. Hoechst 33258 staining of nuclear material was used to double check for the 7-AA-D staining (red emission). Then, a Zeiss LSM-510 Meta confocal laser scanning microscope (Germany) equipped with 63× plan-apo objective was used for further detection of centrosomes, microtubules and chromosomes. The 488 nm argon ion, 543 nm green He-Ne, 633 nm red He-Ne laser lines were used to excite microtubules, nuclei and centrosomes, respectively. Single optical sections (5 μm in thickness) and approximately 45 serial images, from each oocyte on a *z*-axis at 2 μm intervals, were collected by the LSM-510 software (Germany) running on a Siemens–Nixdorf workstation. The final 3D reconstructions and direct measurements on 3D images were performed using Zeiss LSM 510 v3.2 software (Germany).

Staging and scoring of oocytes

Oocyte staging and scoring were carried out by the criteria previously published elsewhere (Albertini, 1992; Can and Albertini, 1997; Can *et al.*,

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2003a) using DIC and multichannel confocal fluorescent detection protocol. Briefly, extrusion of the first polar body, which is best detected by DIC, was used as the main indicator of the M-II stage, whereas the other stages were primarily distinguished by the aid of fluorescently labelled meiotic spindle morphology. In experiments relating to the kinetics of *in vitro* maturation, the meiotic stage was determined by analyzing the chromatin/chromosome distribution pattern after the oocytes had been fixed and stained with Hoechst 33258 dye.

Six percent of all isolated oocytes showing GV-stage chromatin morphology did not resume meiosis, even in control groups. In the BPA group, the number of oocytes that remained at GV-stage was not significantly different from controls. Therefore, all GV-stage oocytes remaining both in control and in BPA-treated cultures were considered as incompetent to resume meiosis and were excluded from the study.

Hydration of the perivitelline space, ruffling and retraction of the oolemma and the darkening of the ooplasm are considered as the signs of a non-vital oocyte. The lack of proper fluorescent staining in such oocytes confirmed that they were not healthy.

Differences among percentages of cells were evaluated by Kruskal–Wallis variance analysis. When the *P*-value from the Kruskal–Wallis test statistics was statistically significant, the multiple comparison test was used to learn which groups differed from the others.

Results

BPA delays meiotic progression

The effects of BPA on meiotic cell cycle progression were evaluated by treating COCs for different time intervals during *in vitro* meiotic maturation process. COCs were exposed to 10 or 30 μM of BPA for either 0–8 or 8–18 h of culture (Figure 1), during which *in vitro* oocyte maturation in mice is normally completed. Figure 2 summarizes the results related to the maturation kinetics during meiosis-I (Figure 2A), early meiosis-II (Figure 2B) and the percentages in each meiotic stage are indicated.

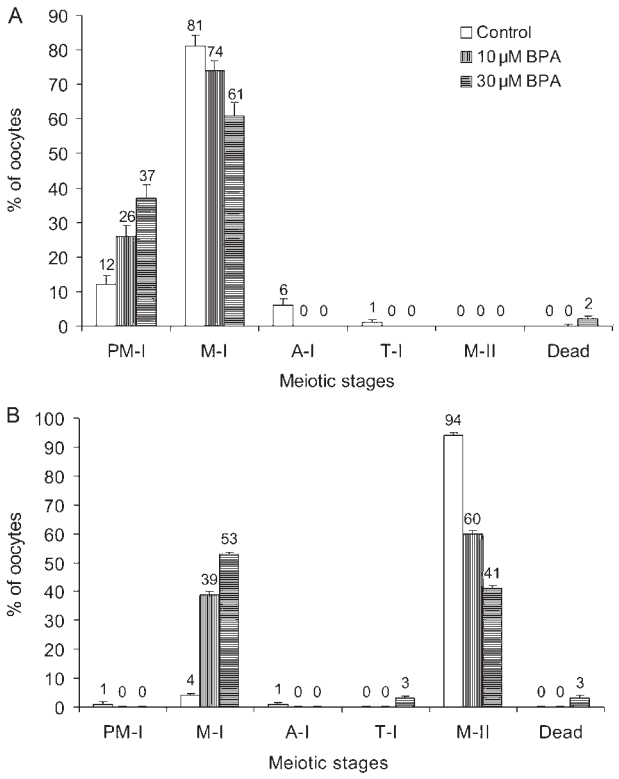

Figure 2. Mitotic indices in control, 10 and 30 μM BPA exposures. (**A**) Represents percentage of cells (*y*-axis) after cells were cultured for 8 h in control (line 1 in Figure 1) and BPA-containing media (line 3 in Figure 1). Note the higher ratio of prometaphase-arrested cells in BPA groups compared to controls. (**B**) Represents percentage of cells cultured for 18 h in control (line 2 in Figure 1) and in BPA after reached to metaphase-I in control medium (line 4 Figure 1). Note the BPA-treated cells in meiosis-I, while 94% of control cells reach to MII. PM-I, prometaphase-I; MI, metaphase-I; A-I, anaphase-I; T-I, telophase-I; MII, metaphase-II.

In general, BPA caused a dose-dependent retardation/inhibition of meiotic progression compared to control cells. During the first half of meiotic division (meiosis-I), as shown in Figure 2A, 10 μM BPA-treated cells showed a slight delay in progression, and they mostly reached the stage of M-I (74%, $P < 0.05$). Only 26% of cells remained in prometaphase ($P < 0.005$). No cell was detected either in earlier stages, such as germinal vesicle breakdown (GVBD), chromatin condensation (CC), or in the later stages, such as anaphase and telophase. In the 30 μM BPA group, 61% of cells reached M-I ($P < 0.001$) while 37% remained in prometaphase-I ($P < 0.005$). Two per cent of all cells treated with 30 μM BPA lost all vitality.

When cells were exposed to BPA during the transition from M-I to M-II (Figure 2B), which normally lasts approximately, 8–10 h in mice, they were apparently found to have become retarded in M-I. A definite amount (39%, $P < 0.001$ and 53%, $P < 0.001$) of cells remained in M-I with 10 and 30 μM BPA exposure, respectively, (94% in controls). Insignificant counts of cells were found in anaphase or were dead.

BPA interferes with centrosomes and perturbs meiotic spindle formation during meiosis-I and II

Retardation or inhibition of meiotic progression led us to assess, whether BPA interferes with cell division machinery or not. Centrosomal protein, pericentrin, which mainly mediate the microtubule anchoring at the centrosome and spindle microtubules were selected as potential targets of BPA as bearing the key roles in certain cell cycle checkpoints. A series of fluorescently labelled antibodies and dyes were used to visualize the entire meiotic spindles elongating from poles to chromosomes as well as microtubule organizing centres (MTOCs) located at spindle poles and throughout the ooplasm. Control PM-I oocytes were characterized by *de novo* synthesis of the first meiotic spindle, though in a non-polarized form (monopolar spindle) in the centre of the oocyte (Figure 3A). Pericentrin was restricted to multiple, centrally located spots where microtubules were emanating. M-I spindles displayed the typical barrel shape slightly translocated to the periphery of the cell (Figure 3B). Chromosomes were aligned around mid-plane where they were tightly bound to kinetochore microtubules. The long axis of the spindle, which transverses the spindle poles, was always perpendicular to the adjacent plasma membrane. The mean distance from pole-to-pole was found to be 29.7 ± 0.9 μm in control M-I oocytes (see Table I for morphometric measurements of M-I and M-II spindles in all experiments). Pericentrin was oriented at spindle poles forming two symmetrical crescents (Figure 3B). Regarding the microtubule mass and interpolar distance (25.1 ± 1.1 μm), M-II spindles were found slightly smaller than M-I spindles (Figure 3C), yet they maintained their barrel shape appearance. They were often found rotated compared to M-I spindles, so that the long axis became nearly parallel to the adjacent plasma membrane. Compared to M-I centrosomes, pericentrin was similar in appearance but in smaller amounts. Interestingly, no pericentrin positivity was noted in the polar body. The spindle dynamics summarized above are considered as normal events that are required for proper meiotic progression (Carabatsos *et al.*, 2000).

In 10 μM BPA-treated cells, a typical PM-I cell was characterized by a newly forming, monopolar spindle. Pericentrin was found

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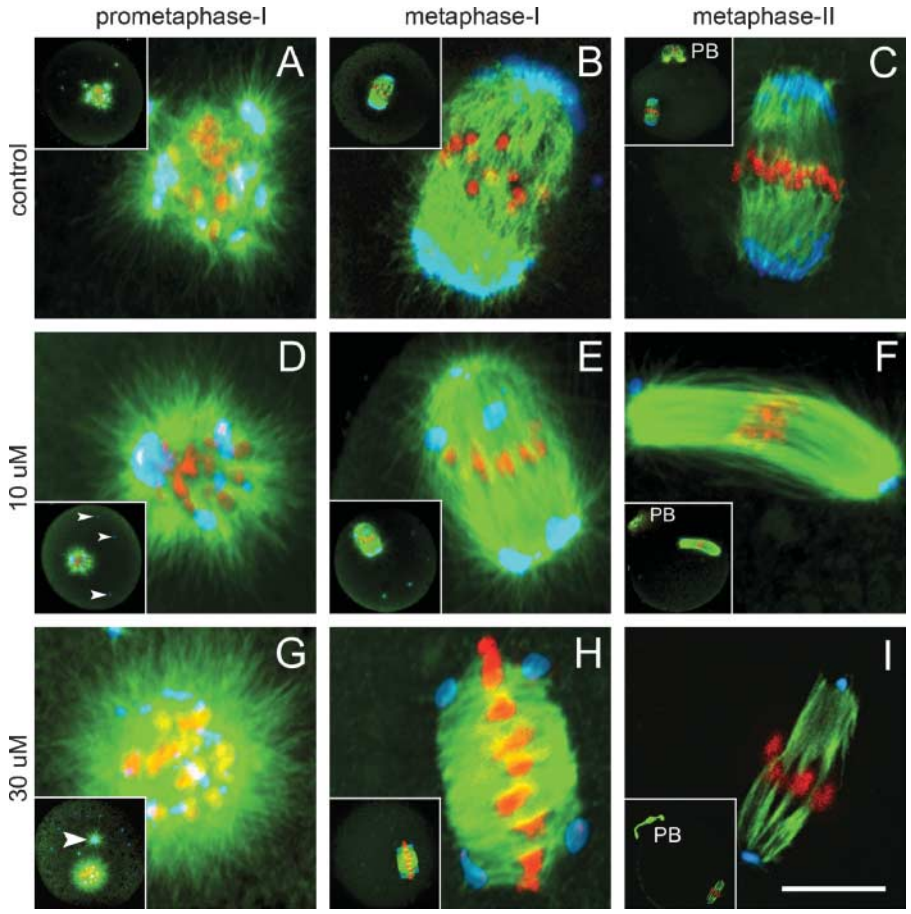

Figure 3. Three dimensional confocal microscopic views of control (**A**–**C**) and BPA-treated (**D**–**I**) oocytes (inlets) and spindles. Triple labelled meiotic spindles stained with antibodies against $\alpha + \beta$ tubulin (1:1) (green); pericentrin (blue) and chromosomes (red) with co-distribution (overlap) depicted in yellow. Inlets show the location of the spindle(s) in the oocyte. A representative prometaphase-I spindle (**A**) with microtubules radiating from centrally located pericentrin foci coalescence at seven-eight major sites that are loosely associated with chromosomes. The spindle at metaphase-I (**B**) has a typical barrel shape with pericentrin symmetrically distributed at the spindle poles as two crescents. Prior to MII, half of the chromosomes (not quantitated) and tubulin mass is expelled from the oocyte to form the first polar body (PB) (**C**). Second meiotic spindle was slightly smaller in size (see also Table I for comparison). A lesser amount of pericentrin is found similar to the one in MI spindle. 10 μM BPA-treated prometaphase-I oocyte (**D**) shows a mass of tubulin, percentrin and chromosomes at the centre while small foci of pericentrin is also noted throughout the ooplasm (arrowheads). In M-I oocytes (**E**) lateral spindle microtubules tend to radiate peripherally yielding a loosened-spindle phenotype. Condensed foci of pericentrin displace from both poles and scatter along the spindle microtubules (compare with **B**). Cytoplasmic pericentrin foci (inlet) are likely to polymerize microtubules. No significant displacement of chromosomes from mid-plane is detected. A 10 μM BPA-treated M-II oocyte (**F**) demonstrates an elongated spindle (see also Table I) associated with a few condensed spots of pericentrin located at each pole. No cytoplasmic pericentrin is noted at this stage. In 30 μM BPA-treated prometaphase-I cells (**G**), increase in number of both centrally and peripherally located (inlet) pericentrin foci is noted (compare with **D**) displaying a big, peripherally located monopolar spindle. A huge spot of accessory pericentrin focus (arrowhead) is occasionally encountered. M-I oocytes display (**H**) remarkable deformations of the spindle. Consistently, they are found compressed from poles so as to enlarge at lateral sides leaving condensed pericentrin foci at four corners. Finally in M-II oocytes (**I**), prominent decrease in size and microtubules mass of the spindle is noted. Note the lack of any chromosome staining in BPA-treated polar bodies in **F** and **I**. Bar represents 10 μm.

associated with the spindle and also dispersed throughout the peripheral ooplasm (arrows in Figure 3D inlet). However, those satellite foci did not polymerize any microtubules.

The prominent feature of M-I spindle poles was the scattering of pericentrin between the mid-plane and the spindle poles (Figure 3E). Spindle microtubules tend to displace especially from the lateral sides of the spindle without drastically altering the spindle dimensions (length = 29.1 ± 1.4 μm; width = 19.3 ± 1.9). Cytoplasmic pericentrin remained at the peripheral ooplasm and began to polymerize microtubules.

The second spindle was found significantly altered (Figure 3F) when oocytes were treated with 10 μM BPA during a critical period at which the first meiotic spindle is partially depolymerized and the second meiotic spindle is formed. The most common form of spindle abnormality (84% of cells) was the elongation of spindle (interpolar distance; 33.3 ± 1.5 μm). Interestingly enough, two condensed spots of pericentrin were noted at each spindle pole (Figure 3F). Despite these alterations regarding the spindle microtubule orientation and PCM, chromosomes were usually found to locate properly at the mid-plane (Figure 3F). Careful examination of all spindles showed no sign of any damage on kinetochore-microtubule junctions. Interestingly, however, an insignificant proportion (2.6%) of polar bodies (PB in Figure 3F) possesses chromosome pairs, implying that all chromosomes were retained in the M-II oocytes.

A 30 μM BPA concentration was then tested before and after the formation of the first meiotic spindle. As expected, a higher rate of atypical oocytes was detected in this group. In PM-I oocytes, pericentrin was found to be extremely dispersed throughout the ooplasm, retaining very few pericentrin foci in association with the spindle (Figure 3G). Occasionally (in 26% of cells), one big pericentrin spot with a significant microtubule polymerization activity

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Table I. Morphometric measuments of meiotic spindles

Groups	Stages	Length (μm) (pole-to-pole)	Width (μm) (equatorial plane)
Control	M-I	29.7 ± 0.9	16.9 ± 0.4
	M-II	25.1 ± 1.1	13.2 ± 0.5
10 μM BPA	M-I	29.1 ± 1.4	19.3 ± 1.9
	M-II	33.3 ± 1.5	8.9 ± 1.0
30 μM BPA	M-I	14.2 ± 2.9	24.8 ± 4.5
	M-II	17.5 ± 3.1	5.5 ± 1.9

Measurements are obtained directly from the digitized 3D reconstructed confocal images using a high-precision measurement tool embedded in the software. Note the variations in both length and width of M-I and M-II spindles exposed to 10 and 30 μM BPA.

was detected close to the monopolar spindle (arrowhead in Figure 3G inlet). Similar to the centrosomes in 10 μM group, dispersed pericentrin foci (cytoplasmic MTOCs) did not polymerize any microtubule.

Quite interestingly, a majority of the M-I oocytes (88% of cells) displayed a widened spindle (length = 14.2 ± 2.9 μm; width = 24.8 ± 4.5) at the lateral axis and were compressed from the pole axis (Figure 3H). Neither microtubule loss nor microtubule-chromosome misconnection was detected. In contrast, severe loss and compaction of the retained pericentrin was apparent and restricted to the corners of those widened spindles (Figure 3H). In M-II oocytes, a lesser amount of pericentrin was observed at the poles (Figure 3I) compared to control or 10 μM BPA-treated M-II oocytes. Centrosome proteins were coupled with a significant loss in spindle microtubule mass that gave rise to the formation of small and loosely oriented M-II spindles (74% of cells) (Figure 3I; compare Figure 3C, F and I). None or very few chromosomes (1.2%) were found in the polar body. Although no karyotype analysis was applied, together with the results found in 10 μM group, this phenomenon implies that BPA somehow causes chromosomes not to segregate equally during meiosis-I and likely to lead to the formation of hyperploidic M-II oocytes even in low doses.

Oocytes hardly recover when freed from BPA exposure

To determine whether the adverse effects of BPA on cell cycle progression and the organization of centrosomes and spindles are reversible, oocytes were initially treated with 10 and 30 μM BPA for 8 h then washed and transferred into control maturation medium for an additional 10 h (see Figure 1, line 5 for experimental design). During 18 h of total incubation, 93% of control cells progressed to M-II (Figure 4). More than half of the oocytes (58%, $P < 0.001$) exposed to 10 μM BPA were able to progress up to M-II while smaller ratio of cells (23%, $P < 0.001$) accumulated in anaphase-I or telophase-I (Figure 4). The remaining 30% of cells were found at prometaphase-I or metaphase-I. A lower rate of cells (38%, $P < 0.001$) reached M-II when treated with 30 μM BPA (Figure 4). The remaining 62% of cells distributed throughout meiosis-I mostly accumulated at M-I (34%). As compared to controls, a higher ratio of cells were detected at anaphase-I and more strikingly at telophase-I during which, normal cells are rarely found. This indicates that BPA causes a true delay and directly interacts with the meiotic machinery at certain checkpoints, therefore, transition from M-I to M-II is taking more time than in controls.

3D confocal images taken from recovered oocytes showed that pericentrin foci tend to reorganize equally at both spindle poles even though the formation of two pericentrin crescents as in controls

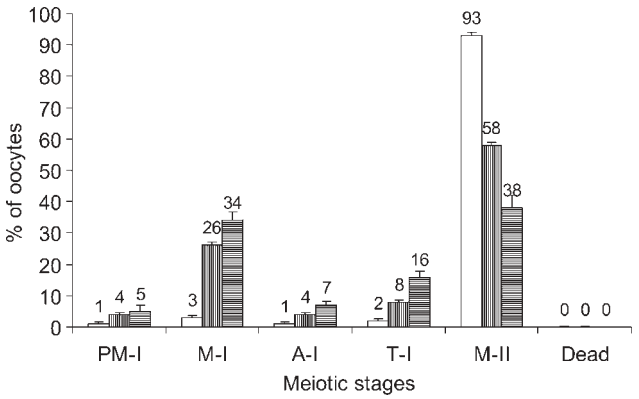

Figure 4. Distribution of oocytes after recovered from BPA exposure. Note that 70% and 61% of cells treated with 10 and 30 μM BPA, respectively, exceed M-I to resume meiosis. Relatively lower ratio of cells in anaphase-I and telophase-I indicates that recovery time in control medium (10 h) is not adequate for the completion meiosis-II. See Figure 2 for abbreviations.

have not been completed. Although centrosomal proteins seemed to recover to a certain extent, microtubules and attaching chromosomes were found poorly reorganized (compare Figure 3E with Figure 5A). M-II oocytes, on the other hand, gave better results in terms of pericentrin localization, microtubule and chromosome reorganization (Figure 5B). Chromosome distribution in the polar body was significantly restored in those cells compared to unrecovered groups (compare red signals in polar bodies in Figures 3F, I and 5B).

Discussion

The present studies were designed to evaluate the effects of a widely used industrial chemical, BPA, on the process of meiotic cell division machinery in cultured mouse oocytes, as a possible model for determining the mode of action of estrogenic agents on meiosis in mammalian germ cells. Although examining the *in vitro* actions of estrogenic agents on oocytes may not accurately reflect their *in vivo* activity, there is mounting evidence to suggest that BPA and related compounds impair reproductive function in mammals (Caroline *et al.*, 2003) by a potential action that interferes with meiosis at key stages of germ cell nuclear maturation (Hunt *et al.*, 2003). Given the multistep nature of meiosis and the stage-specific involvement of the cytoskeleton in general, centrosomal proteins and microtubules in particular (Carabatsos *et al.*, 2000), the present studies were undertaken, for the first time in the literature, to evaluate the actions of BPA during later stages of oocyte maturation.

Although meiotic aneuploidy was reported to be relatively low in mice compared to humans, and the rates of hyperploidy are approximately 0.5–1% (Bond and Chandley, 1983), increasing amount of data are emerging from experiments in mice that certain environmental agents possess potent meiosis-disrupting capability that directly interferes with the cell division machinery both in meiosis-I and early meiosis-II (Can and Albertini, 1997; Can and Semiz, 2000). So, mouse oocytes, COCs and meiotic spindles in particular, increasingly gain importance serving as sensitive cellular assay systems for the study of reproductive toxins. We now carefully extend our results to humans. Nonetheless, meiosis is a highly conserved process among mammalian organisms. Such an *in vitro* assay in which isolated gamete cells are tested in controlled time and dose experiments will demonstrate isolated cell responses to drugs no matter the origin of the cells.

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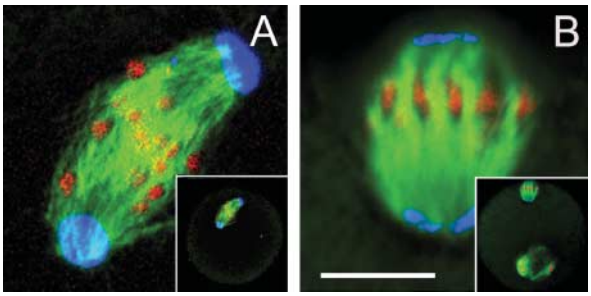

Figure 5. Three-dimensional confocal microscopic views of the recovery of oocytes after 30 μM BPA. A representative M-I oocyte (**A**) is characterized by the restoration of pericentrin foci (blue) at spindle poles remaining a few spots in the ooplasm. However, the overall reorganization of spindle microtubules (green) and chromosome (red) alignment are yet to be recovered. A relatively fully recovered M-II oocyte (**B**) is shown with well-organized pericentrin protein at spindle poles, MII spindle microtubules and equatorially aligned chromosomes. Note the relocation of chromosomes in polar body. Bar represents 10 μM.

The hyperploidic phenotype of oocytes reported by Hunt *et al.* (2003) due to an inadvertent *in vivo* exposure to BPA lends credence to the results in this study that 5.8% of overall oocytes were found hyperploid after long-term oral BPA exposure in laboratory mice during caging conditions. The relatively small ratio of hyperplodic cells in their study is most likely related to the differences of the administration route of BPA. Our isolated cell culture experiments led to a higher ratio of chromosome misalignment compared to their *in vivo* BPA exposures, since the BPA dose in our study is approximately 100 times higher than their *in vivo* doses.

How and when does BPA lead to meiotic aneuploidy? Certainly slight perturbations in spindle organization could underlie problems in chromosome segregation at either meiosis-I or -II, and the abnormalities in polar body extrusion noted previously (Can and Albertini, 1997) could be a consequence of subtle changes in meiotic spindle organization. An estimated 10–25% of fertilized human oocytes are aneuploid (Hassold and Hunt, 2001), and since the vast majority (~75%) of human aneuploidies originate during meiosis-I (Gaulden, 1992), a particular effort has been directed in this study towards elucidating the centrosome and microtubule dynamics during meiosis-I and partly meiosis-II, two critical periods during which the BPA could differentially act. Critical exposure period of BPA remains as the main concern to be elucidated after the study of (Hunt *et al.* 2003). So, the experiments in this study were designed to investigate the differential effects of BPA at two consecutive periods of meiosis. Results revealed the consequences of temporal exposure of BPA. Due to the data obtained, a second meiotic spindle is found more vulnerable to BPA than first meiotic spindle at a given dose. While only centrosomal proteins were spatially misaligned in the first period, the integrity of centrosomes was dramatically altered and the amount of spindle microtubules diminished during the second period. Given the timing of meiosis in mammals, it is therefore possible to hypothesize that even acute exposure to BPA exerts its effects by interfering with cellular components in a relatively short period of time.

In addition to the differential effects of BPA on different phases of meiosis, the results obtained here demonstrate that the spindle and related structures in meiotic cells are more sensitive to BPA than in mitotic cells (Pfeiffer *et al.*, 1997; Nakagomi *et al.*, 2001). Despite studies carried out on mitotic cells (Pfeiffer *et al.*, 1997; Nakagomi *et al.*, 2001) or on cell-free systems (Pfeiffer *et al.*, 1997; Xu *et al.*, 2002), no study has been reported so far that evaluates *in vitro* BPA toxicity on meiotic cells. Our experiments showed that much lower doses are sufficient to disrupt the spindle pole integrity and microtubules in meiotic cells compared to mitotic cells. Pfeiffer *et al.* (1997) and Nakagomi *et al.* (2001) reported that mitosis is arrested at metaphase through microtubule interactions by 200 μM BPA or higher doses in Chinese hamster V79 mitotic cells. Only cell-free systems or other than cytoskeletal elements (apoptotic proteins) are found receptive to femto or pico molar BPA exposures (Xu *et al.*, 2002). Taken together, one can postulate that BPA is apparently more potent in gametes than in somatic cells and may be considered as a reproductive toxin based on its daily uptake.

In addition to its sensitivity to BPA, using such a unique 2-cell system (i.e. COC) also proved to be a proper paradigm for testing estrogenic drugs since COCs in mouse (Hiroi *et al.*, 1999) and human (Palter *et al.*, 2001) were demonstrated to express both types of estrogen receptor (ER), i.e. ERα and ERβ, and found to be very sensitive to BPA (Xu *et al.*, 2002; Kurosawa *et al.*, 2002). In view of the fact that granulosa cells and particularly cumulus cells directly link to the metabolic and structural growth of the enclosing oocyte (Albertini *et al.*, 2001), from the perspective of reproductive toxicology, exposure to BPA will eventually affect reproductive outcome by interfering with follicular development as well.

Both ER subtypes are influenced by BPA treatment in a varying fashion (Hiroi *et al.*, 1999). Hiroi *et al.* (1999) demonstrated that the estrogenic activity significantly decreased when 293T and Hec-1 cell lines expressing ERα were incubated with 10 μM BPA in the presence of 0.1 μM 17β-estradiol while the activities mediated by ERβ were unchanged in the same conditions. They concluded that BPA only acts as an agonist of estrogen via ERβ, whereas it has dual actions as an agonist and antagonist in some types of cells via ERα. On the other hand, the adverse effects of BPA have been shown to occur not only through ER receptors, but also by a direct disruptive action on microtubules (Pfeiffer *et al.*, 1997). Moreover, we have recently observed the direct binding of BPA to taxol-stabilized tubulin dimers, and thus an inhibition of tubulin polymerization in cell-free systems (Kiliç *et al.*, 2003). Taken together, it is possible to propose that BPA action on isolated COCs presented here could be due to either direct binding of BPA to tubulin, pericentrin or related molecules or through a mechanism where ERs play autocrine and/or paracrine roles in presenting BPA to the maturing oocyte.

This study shows that adverse effects of BPA on centrosome and/or microtubules are stage- and cell-specific, since both the initial phase of meiotic maturation, involving GV, GVBD, CC and cumulus cells remained intact even at relatively high concentrations of BPA (preliminary studies). Those results led us to assume that a meiosis-disrupting effect of BPA is likely to be related to disorganization in meiotic MTOCs that consequently leads to certain errors in microtubule organization, at least in tested doses. The resistance of interphase centrosomes and microtubules either in GV-stage oocytes or in cumulus cells suggests that meiotic centrosomes, and thus microtubules, are more susceptible to the adverse effects of BPA, and therefore might ultimately result in a series of genetic defects in oocytes such as aneuploidy or fertilization failure without grossly altering the surrounding cells and tissues. Similar results were previously demonstrated using some other estrogenic reagents such as diethylstilboestrol (DES) (Can and Semiz, 2000). Supporting evidence also comes from other studies (Pfeiffer *et al.*, 1997) that different stages of mitosis are differentially affected by BPA. As a result, the possibility of the survival of an oocyte varies depending on the stage that interacts with a meiosis-disrupting agent at a given dose. Pfeiffer *et al.* (1997) demonstrated that cell growth rate falls to 50% with 200 μM BPA treatment, and they suggested that BPA leads to a prolongation of the metaphase period, but not to

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a complete block of the cell cycle. In our study, the observations that meiotic resumption was mainly interrupted at the metaphase-anaphase transition of meiosis-I but not in subsequent anaphase or telophase, supports the hypothesis that BPA is acting at this critical juncture of M-phase. In both mitotic and meiotic systems, anaphase is known to depend on the ubiquitin-mediated proteolysis of cyclins, the regulatory subunit of maturation-promoting-factor (MPF) (Murray and Hunt, 1993). Therefore, BPA might be preventing anaphase onset by inhibiting cyclin degradation. This is a plausible explanation for the action of BPA since protease inhibitors have been shown to arrest somatic cells at metaphase by inhibiting cyclin degradation (Sherwood *et al.*, 1993). Another implication of cell cycle impairment by BPA is reported by Xu *et al.* (2002) who demonstrated that primary cell cultures of murine ovarian granulosa cells were arrested at G_2-M transition exposed to 100 μM BPA for 24–72 h.

A major function of centrosome(s) in an animal cell is to nucleate microtubules. For this reason, centrosomes were particularly chosen for study in order to document the possible causes of spindle abnormalities by us and others. Pericentrin is thought to mediate microtubule anchoring at the centrosome by binding a multiprotein complex that nucleates microtubules, the tubulin ring complex. Functional studies have revealed a key role for pericentrin in microtubule organization, spindle assembly and chromosome segregation (Doxsey *et al.*, 1994). For instance, overexpression of pericentrin causes microtubule defects, chromosome missegregation and aneuploidy (Pihan *et al.*, 2001). Using a pericentrin antibody to visualize centrosomes therefore would be an extremely sensitive microscopy technique to track the centrosome dynamics within cells. Previous studies confirm that pericentrin protein is dynamically expressed in cells reaching its maximally polymerized form at metaphase and quickly depolymerizing to its soluble form just after mitosis (Dictenberg *et al.*, 1998) and assembly of pericentrin reoccurs concomitant to increased microtubule-nucleating activity. The pericentrin pattern in mouse M-I and M-II spindles was demonstrated as a crescent or semi-ring shape at both poles (Carabatsos *et al.*, 2000). In early stages of meiotically competent mouse oocytes, pericentrin was found to be confined to a single large locus adjacent to the nucleus. It reorganized to newly forming multiple MTOCs during CC and GVBD, and finally congregated as 6–9 spots in a monopolar spindle during prometaphase-I (Can *et al.*, 2003a). Interestingly, during telophase, pericentrin is not translocated to the polar body, implying that meiotic division machinery conserves the whole PCM material within the oocyte to be used in meiosis-II. This might also explain the lack of a spindle formation in polar body that fails to divide when the MII oocyte is fertilized.

Protein kinase (PK) family members are known to play crucial roles in the regulation of cell growth, cell cycle progression and cell differentiation (Livneh and Fishman, 1997). PKC I has been shown to co-localize with microtubules and bind microtubule-associated proteins (Kiley and Parker, 1995), novel PKC isoforms and have been reported to localize to the centrosome (Takahashi *et al.*, 2000). PKC-deficient T cells failed to develop a polarized microtubule network, a defect that can be rescued by expressing PKC I (Volkov *et al.*, 2001). Thus, PKC is emerging as a key regulator of microtubule organization. Recently, endogenous PK-C βII was found to directly bind to pericentrin at centrosomes in mitotic cells (Chen *et al.*, 2004). Disruption of this interaction by expression of the interacting region of pericentrin results in release of PKC from the centrosome, microtubule disorganization, centrosome misalignment, a series of disturbances similar to ours. Conclusively, it is supposed that spatio-temporal interaction between PKC and pericentrin has a pivotal role in centrosome function and a series of subsequent cellular reorganization such as microtubule assembly, chromosome segregation and cytokinesis. Therefore, it is possible to suggest that BPA may act on PKC-pericentrin domain that ultimately results in cell cycle delay and aneuploidy. Further studies are needed to elucidate the structural and functional changes in protein kinases, particularly A and C, due to varying doses of BPA exposure in meiosis.

On the other hand, pericentrin assembly requires microtubules and the microtubule motor *dynein* for normal microtubule nucleation and centrosome separation (Robinson *et al.*, 1999). Recent studies demonstrated that reduced levels of pericentrin have a diminished capacity to nucleate microtubules (Young *et al.*, 2000). Moreover, pericentrin has been shown to be a minus-end dynein-mediated cargo along microtubules to be transported to spindle poles (Young *et al.*, 2000). When dynein-ATPase is inhibited by sodium orthovanadate, the oocytes are arrested in metaphase and the pericentrin becomes associated with both spindle poles as multiple solitary condensed aggregates (Carabatsos *et al.*, 2000), in a very similar fashion observed in the present study. Based on the above data, it is possible to suggest that proper transportation of PCM proteins to poles is temporally affected by the interaction of BPA with microtubules and/or dynein that results in a varying degree of centrosome disassembly and dysfunction. Thus, motor proteins and other regulatory mechanisms of transportation along spindle microtubules and cytoplasmic microtubules might be considered as the target sites for BPA and other estrogenic compounds. Recovery experiments support this hypothesis that when cells are transferred into control medium, cells do recover to a certain extent and the pericentrin, which was previously deteriorated by BPA, is the first to recover to its original state. Along with the use of some well-known reversible microtubule drugs, such as nocodazole, colchicine and taxol, it is suggested that BPA might also be used as a chemical tool for understanding the delicate mechanism of meiotic cell division since the minute dose changes could alter the spindle microtubular arrays orchestrated by the centrosomes.

In conclusion, BPA has been shown to act as a meiosis-disrupting agent by selectively interfering with centrosome and microtubule organization. This moderate activity of BPA, unlike other potent estrogenic agents that depolymerize microtubules, seems to be due to centrosome disorganization and fragmentation resulting in poorly organized spindles in both meiotic and mitotic systems (Can A, unpublished observations in Vero cells). Although the precise mechanism of BPA action requires clarification, the prospect that this industrial and dental compound acts as a cell cycle disruptor warrants the pursuit in future studies of human cell culture models using a dynamic live cell analysis involving GFP-pericentrin and/or tubulin. Such an analysis would undoubtedly provide a more precise time scale for changes in centrosome and microtubule array.

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