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Mechanisms underlying disruption of oocyte spindle stability by bisphenol compounds

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Abstract

Accurate chromosome segregation relies on correct chromosome-microtubule interactions within a stable bipolar spindle apparatus. Thus, exposure to spindle disrupting compounds can impair meiotic division and genomic stability in oocytes. The endocrine disrupting activity of bisphenols such as bisphenol A (BPA) is well recognized, yet their damaging effects on spindle microtubules (MTs) is poorly understood. Here, we tested the effect(s) of acute exposure to BPA and bisphenol F (BPF) on assembled spindle stability in ovulated oocytes. Brief (4 h) exposure to increasing concentrations (5, 25, and 50 µg/mL) of BPA or BPF disrupted spindle organization in a dose-dependent manner, resulting in significantly shorter spindles with highly unfocused poles and fragmented pericentrin. The chromosomes remained congressed in an abnormally elongated metaphase-like configuration, yet normal end-on chromosome-MT attachments were reduced in BPF-treated oocytes. Live-cell imaging revealed a rapid onset of bisphenol-mediated spindle MT disruption that was reversed upon compound removal. Moreover, MT stability and regrowth were impaired in BPA-exposed oocytes, with few cold-stable MTs and formation of multipolar spindles upon MT regrowth. MT-associated kinesin-14 motor protein (HSET/KIFC1) labeling along the spindle was also lower in BPA-treated oocytes. Conversely, cold stable MTs and HSET labeling persisted after BPF exposure. Notably, inhibition of Aurora Kinase A limited bisphenol-mediated spindle pole widening, revealing a potential interaction. These results demonstrate rapid MT disrupting activity by bisphenols, which is highly detrimental to meiotic spindle stability and organization. Moreover, we identify an important link between these defects and altered distribution of key spindle associated factors as well as Aurora Kinase A activity.

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Introduction

Accurate chromosome segregation during meiotic division depends on the assembly of a bipolar spindle apparatus and formation of correct chromosome-microtubule (MT) interactions (Chmátal *et al.* 2015). Thus, exposure to spindle disrupting compounds can promote meiotic errors, which in turn lead to aneuploidy in oocytes and genomic instability in developing embryos upon fertilization (Nagaoka *et al.* 2012). The adverse effects posed by environmental toxicants on gamete quality and function is a growing concern. In the current study, we tested the effect of bisphenol compounds, including bisphenol A (BPA) and bisphenol F (BPF), on assembled meiotic spindle organization in ovulated MII oocytes, a critical stage prior to fertilization.

Biphenol A, widely used in plastics manufacture, is recognized as a potent endocrine disruptor with estrogen-like activity as it can bind to intracellular (ERα, ERβ) and cell surface (GPR30) estrogen receptors to affect cell signaling and proliferation (Acconcia *et al.* 2015, Gore *et al.* 2015). Increasing evidence supports that BPA has significant detrimental effects on human and animal health (Gore *et al.* 2015, Pelch *et al.* 2019), which has prompted some restrictions on its use. Yet, it remains highly prevalent. Moreover, the use of replacement analogs, such as bisphenol S (BPS) and bisphenol F (BPF), has increased in recent years. However, these compounds are structurally similar to BPA and also pose potential safety concerns (Sartain & Hunt 2016, Pelch *et al.* 2019). Studies demonstrate that BPA adversely affects various aspects of the reproductive system in males and females, including germ cell development and function (Peretz *et al.* 2014). In females, maternal exposure to environmentally relevant doses of BPA has been shown to disrupt early ovarian follicle development (Hunt *et al.* 2012, Berger *et al.* 2016) and promote increased rates of oocyte meiotic abnormalities, including aneuploidy (Hunt *et al.* 2003, Pacchierotti *et al.* 2008). Meiotic abnormalities in oocytes are also evident, following *in vitro* ovarian culture with continuous exposure to BPA (Lenie *et al.* 2008).

While the endocrine disrupting activity of BPA is well documented, studies also point to damaging effects on microtubules (MTs) that are less understood.

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Microtubule and spindle defects have been reported in response to BPA exposure during mitotic division in diverse systems, including somatic cells lines (Pfeiffer *et al.* 1997), plant cells (Adamakis *et al.* 2019), and sea urchin embryos (George *et al.* 2008). Meiotic abnormalities in mammalian oocytes following maternal *in vivo* exposure (Hunt *et al.* 2003) or ovarian follicle culture (Lenie *et al.* 2008), with BPA were frequently associated with meiotic spindle MT disruption. Notably, exposure to BPA or common replacement analogs during *in vitro* meiotic maturation has been shown to hinder the progression to metaphase-II (MII) as well as disrupt meiotic spindle assembly and organization in mouse (Can *et al.* 2005, Eichenlaub-Ritter *et al.* 2008), porcine (Zalmanova *et al.* 2017), bovine (Campen *et al.* 2018), and human (Machtinger *et al.* 2013) oocytes. Nevertheless, the underlying mechanisms of bisphenol-mediated spindle MT disruption in oocytes remain poorly defined. Oocytes exposed to bisphenol during meiotic maturation often fail to progress to MII, making it difficult to discern between potential direct effects on spindle MTs and disruption of meiosis. Moreover, many investigations used cumulus cell-enclosed oocytes when testing BPA exposure *in vitro*, raising the question as to whether meiotic abnormalities might also be partially attributed to bisphenol effects on the surrounding granulosa cells (Peretz *et al.* 2014, Mansur *et al.* 2017).

In the current study, we exposed ovulated oocytes to BPA and a common replacement analog, BPF, to gain a better understanding of the direct effect(s) of bisphenols on MTs and spindle organization. The aim was to test the acute impact of bisphenols on assembled MII spindle organization and stability rather than the regulation of initial spindle assembly. Our results demonstrate rapid MT-disrupting activity, by BPA and BPF, which is detrimental to oocyte spindle organization and stability as well as spindle-associated factors. Moreover, we show differences in BPA and BPF action on MTs, as well as identify an important link between bisphenol-mediated MT disruption and Aurora Kinase A activity.

Materials and methods

Chemicals

Bisphenol A (BPA) and Bisphenol F (BPF) were dissolved in DMSO to prepare stock concentrations. Single use aliquots were frozen until use and subsequent dilution in culture media to prepare the test concentrations. All chemicals were obtained from Sigma-Aldrich unless otherwise indicated.

Animals

C57BL/6J female and DBA/2J male mice were purchased from the Jackson Laboratory (Bar Harbor, ME) for breeding. All mice were housed in individually ventilated cages at a constant temperature (24–26°C) and under a controlled light cycle (12 h light:12 h darkness) with food and water provided *ad libitum*. All animal use protocols were approved by the 'Institutional Animal Care and Use Committee' (IACUC) at the University of Georgia, and the experiments were conducted in accordance with all specified guidelines.

Ovulated oocyte collection and culture with bisphenol compounds

Oocytes were obtained from B6D2F1 mice (C57BL/6J females × DBA/2J males) for all studies. To promote ovarian follicle development, 20- to 21-day-old female mice were treated with 5 IU PMSG (EMD Biosciences, La Jolla, CA). Approximately 48 h later, 5 IU of hCG (EMD Biosciences) were administered to induce the ovulation of mature metaphase-II (MII) oocytes. Oocytes were recovered from the oviducts 16 h post hCG treatment in Minimal Essential Medium (MEM) with 3 mg/mL BSA, then briefly transferred to media supplemented with hyaluronidase to remove the surrounding cumulus cells. The denuded MII oocytes were then cultured for 4 h in MEM/BSA supplemented with increasing concentrations (5, 25, and 50 µg/mL) of BPA or BPF, based on earlier studies in human and mouse oocytes (Machtinger *et al.* 2013, Nakano *et al.* 2016). Control oocytes were similarly cultured in MEM/BSA with 0.5 µL/mL DMSO. All cultures were maintained at 37°C with 5% CO_2, 5% O_2, and 90% N_2. After the 4 h incubation, all oocytes were fixed for analysis. A total of three to four replicates were carried out, with 40–50 oocytes per treatment group. Subsequent experiments tested whether potential bisphenol effects are reversed upon compound removal. In brief, ovulated oocytes were exposed to BPA or BPF (25 and 50 µg/mL), as previously described, then transferred to fresh MEM/BSA to wash off the compound and cultured for an additional 4 h prior to fixation.

Immunofluorescence analysis

Oocytes were fixed and immunolabeled with specific antibodies as previously described (Baumann & Viveiros 2015), including anti-TPX2 (1/500; Novus Biological, Centennial, CO), anti-PCNT (1/1000; BD Biosciences, San Jose, CA or Covance, Princeton, NJ), anti-acetylated α-tubulin (1/1000; Sigma-Aldrich), and anti-pT288AURKA (1/1000; Cell Signaling Technology). In brief, the fixed oocytes were incubated with the primary antibodies overnight at 4°C, then washed and successively incubated (1 h) at 37°C with specific Alexa Fluor conjugated 488 or 555 secondary antibodies (Life Technologies). After a final wash, the oocytes were transferred onto glass slides and overlaid with mounting medium (Vecta Shield, Vector Laboratories, Burlingame, CA) containing DAPI to counterstain the DNA. To detect Kinesin-14 motor protein, HSET/KIFC1, in oocytes the zona pellucida (ZP) was removed by a brief exposure to acid Tyrodes solution. Zona-free oocytes recovered for 5 min in MEM/BSA at 37°C, then were fixed directly onto glass slides with 4% PFA with 0.1% Triton X-100 in ddH_2O at room temperature. The oocytes were labeled, as previously described, with anti-acetylated α-tubulin (1/1000; Sigma-Aldrich) and anti-KIFC1 (1/50; Novus Biologicals). Spindle structure as well as fluorescence (PCNT, TPX2,

α-tubulin, and pT288AURKA) were assessed in a nonblinded manner, using a Leica DMRE upright fluorescent microscope with imaging software (Leica Microsystems) or Zeiss LSM710 equipped with a 40x oil emersion lens. In addition, HSET/KIFC1 distribution was assessed by high resolution structured illumination microscopy (SR-SIM) using a Zeiss Elyra S1 system equipped with 100x oil immersion lens, and ZEN 2011 software with a SIM analysis module was used for image acquisition at the Biomedical Microscopy Core (BMC) facility, University of Georgia.

Live cell imaging

Meiotic spindle microtubule dynamics in BPA and BPF-exposed oocytes were assessed by live cell imaging and compared to controls. Time-lapse image acquisition was performed following microinjection of a capped mRNA (cRNA) cocktail encoding histone RFP-H2B and EGFP-MAP4 fusion proteins into the cytoplasm of fully-grown prophase-I arrested (GV-stage) oocytes in order to visualize the chromosomes (red) and microtubules (green), respectively (Baumann *et al.* 2017). Following cRNA microinjection, the oocytes were cultured for 17 h at 37°C in MEM/BSA to enable sufficient recombinant protein expression for imaging and oocyte maturation to MII. After culture, MII-stage oocytes were transferred to a 200 µL micro-drop of MEM/BSA with 50 µg/mL BPA or BPF. These drops were maintained under oil in an environmental chamber. Meiotic spindle pole organization and microtubule dynamics were monitored for 4 h by time-lapse microscopy at 15 min intervals using a Nikon Eclipse Ti-U/D-Eclipse C1 laser scanning confocal microscope equipped with a 40x objective lens. Image acquisition was conducted using EZ-C1 software (Nikon) with a step size of 5 µm and a Z-stack range of 100 µm. Imaging data were analyzed by maximum intensity and 3D reconstructions using NIH Elements software (Nikon).

Assessment of kinetochore-microtubule (MT) attachments

Chromosome-MT attachments were compared between control, BPA, and BPF-treated oocytes as previously described (Baumann *et al.* 2017). Ovulated MII oocytes were cultured for 4 h in MEM/BSA alone or media with 50 µg/mL of BPA or BPF. Following culture, the oocytes were cold treated at 4°C in M2 Media for 10 min to depolymerize unstable MTs, then fixed immediately with 2% PFA/0.1% Triton X-100 for 30 min at 37°C. The oocytes were then blocked in PBS with 0.1% BSA and 0.1% Triton X-100 for 45 min, prior to immunostaining. Human CREST autoimmune serum (Nuclear ANA-Centrosome Autoantibody) (1/500; Cortex Biochem, Concord, MA) and anti-α-tubulin (1/4000; Sigma-Aldrich) were used to label the kinetochores and spindle MTs, respectively. To visualize the entire meiotic spindle of each oocyte, the slides were assessed using a Nikon Eclipse Ti-U/D-Eclipse C1 laser scanning confocal microscope equipped with a 40x oil immersion lens. EZ-C1 software was used for image acquisition with a step size of 0.3 µm and a Z-stack range of 2–6 µm. Confocal Z-stacks were subsequently analyzed using NIH Elements software to classify kinetochore-MT attachments in each layer.

Microtubule (MT) stability regrowth analysis

Spindle MT stability was tested in response to bisphenol exposure. Ovulated oocytes were cultured for 4 h in MEM/BSA media with 50 µg/mL BPA or BPF, while control oocytes were cultured in media alone. The oocytes were then transferred to cold (4˚C) M2 media for 30 min to depolymerize the spindle MTs, as indicated. After cold treatment one group of oocytes was immediately fixed for analysis. A second group was transferred to pre-warmed M2 media at 37˚C for MT regrowth then fixed at 5 min post warming. The M2 medium was also supplemented with bisphenol (50 µg/mL). Oocytes in all groups were immunolabeled with anti-acetylated α-tubulin and anti-PCNT antibodies. MT organization was analyzed in oocytes using a Leica Microsystem fluorescent microscope with imaging software.

Aurora Kinase A (AURKA) inhibition

Aurora kinase A (AURKA) plays an essential role in meiotic spindle assembly and organization (Solc *et al.* 2012, Bury *et al.* 2017). Therefore, we tested whether meiotic spindle organization in response to BPA or BPF exposure is potentially influenced by AURKA. Ovulated-MII oocytes were collected and exposed to BPA or BPF (25 and 50 µg/mL) during a 4 h culture with or without 500 nM MLN8237 (Selleckchem, Houston, TX), a selective inhibitor for AURKA (Sloane *et al.* 2010). Control oocytes were incubated in media alone under similar conditions. After the 4 h culture, all oocytes were fixed for immunofluorescence analysis to assess pT288AURKA expression as well as chromosome and meiotic spindle configurations as previously described using a Leica Microsystem fluorescent microscope with imaging software.

Western blotting

Oocytes ($n = 50$ per group) were frozen in RIPA buffer supplemented with a protease and phosphatase inhibitor solution. The samples were thawed on ice and mixed with 5x loading buffer, then heated at 98°C for 7 min. Proteins were separated in 10% acrylamide gels, then transferred onto a hydrophobic PVDF membranes (Millipore). The membrane was blocked in TBST supplemented with 2% Tween 20 and 5% FBS for 1 h at room temperature and incubated with anti-pT288 AURKA (1/1000; Cell Signaling Technology) at 4°C overnight. The following day, the membrane was washed in TBST and incubated with a peroxide-conjugated secondary antibody (Jackson ImmunoResearch) for 1 h. ECL (Millipore) was used for chemiluminescent detection. The membrane was also probed with anti-AURKA (1/1000; Novus Biologicals) and anti-β tubulin (1/2000; Sigma-Aldrich) as an internal control, under similar conditions. Individual band intensity was quantified using the ImageJ software and the relative total protein values in each group were compared to the control, which was normalized to 1.0.

Statistical analysis

All data are presented as mean percentages (±S.E.M.) from a minimum of three independent experimental replicates. Scoring was performed in a nonblinded manner. The GraphPad Prism software was used for data analysis and preparation of all graphs. The data were analyzed by either ANOVA for multiple comparisons or *t*-test for comparison among two groups, and differences were considered to be significant when $P<0.05$.

Results

Brief exposure to BPA or BPF disrupts the organization of fully assembled MII spindles

To test the effect of BPA and BPF on assembled meiotic spindle organization (Fig. 1), ovulated MII oocytes were incubated for 4 h in media supplemented with increasing concentrations (5, 25, and 50 µg/mL) of BPA or BPF. Quantitative morphometric analysis showed that 98% of control oocytes contained an organized bipolar spindle with PCNT localized at the spindle poles in a complete or partial ring-like structure and congressed chromosomes (Fig. 1A, a). In contrast, oocytes exposed to BPA contained significantly shorter spindle structures with highly unfocused spindle poles and disrupted PCNT organization (Fig. 1A, b, c and d). Surprisingly, the chromosomes remained congressed in an abnormally elongated metaphase plate-like configuration, and TPX2 distribution along the spindle MTs was seemingly unaffected. BPA-mediated spindle pole disruption occurred in a dose-dependent manner (Fig. 1B). The majority of oocytes exposed to higher BPA levels contained very short spindles with extremely unfocused poles, a subset (~44%) of which also showed separated/splayed microtubules at the poles (Fig. 1A, d inset). Exposure to higher BPF concentrations also promoted a significant increase in the percentage of oocytes (~36%) with unfocused spindle poles, although not as wide compared to the BPA-treated groups. Morphometric analysis revealed significantly reduced spindle length (pole-to-pole distance), but increased spindle pole width in all BPA groups and the highest BPF concentration (Fig. 1C and D). The ratio of spindle pole width to spindle length was markedly increased in response to higher BPA and BPF concentrations (Fig. 1E). These data establish that even a brief (4 h) exposure

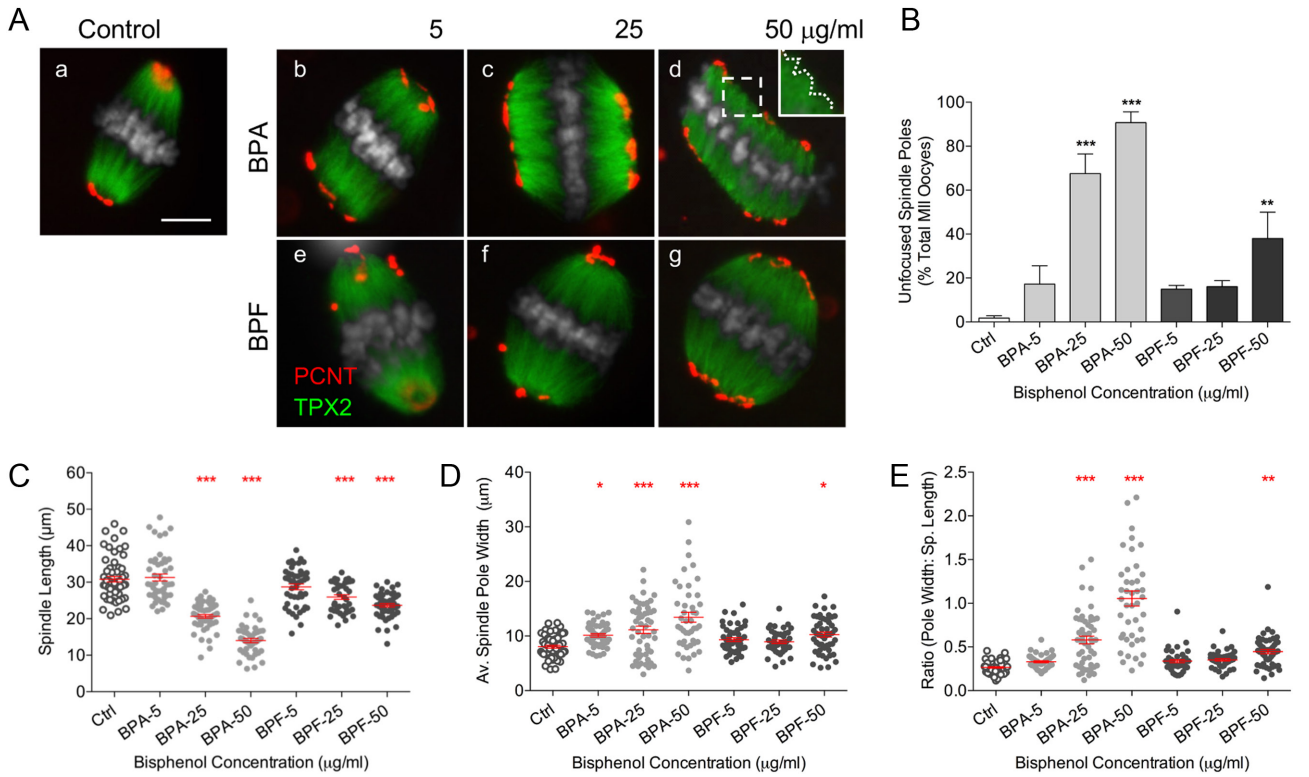

Figure 1 Brief exposure to BPA or BPF disrupts the organization of assembled meiotic spindles in ovulated metaphase-II (MII) mouse oocytes. (A) Meiotic spindle organization in oocytes exposed to increasing concentrations (5, 25, and 50 µg/mL) of BPA (b, c, and d) or BPF (e, f, and g) during a 4 h culture, relative to control oocytes (a) in media alone. The oocytes were double-labeled with anti-TPX2 (green) on microtubules and anti-pericentrin (red) antibodies. DAPI labeled DNA is shown in grey. (B) Percent (mean ± S.E.M.) total oocytes with unfocused (broad) meiotic spindle poles from three replicates (30–40 oocytes/group). Quantitative analysis (mean ± S.E.M.) of the (C) meiotic spindle length (pole-to-pole distance), (D) average spindle pole width, and (E) ratio of the pole width to spindle length measured in control (open circles), BPA (grey circles), and BPF-exposed (black circles) oocytes (40–60 total per group) from three replicates. Scale bar: 10 µm. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ compared to the control group.

to either BPA or BPF is highly disruptive to assembled meiotic spindle organization.

Bisphenol compounds fragment PCNT at spindle poles and disrupt chromosome-MT attachments

In bisphenol-treated oocytes, meiotic spindle disruption was associated with fragmented distribution of PCNT, a key aMTOC scaffolding protein (Baumann *et al.* 2017), at the spindle poles (Fig. 2). Over 90% of control oocytes showed PCNT clustered at the spindle poles either in a complete or partial ring-like configuration (Fig. 2A, a, b and c). However, PCNT was detected in a highly fragmented/dotted pattern along the unfocused meiotic spindle poles in BPA-treated oocytes (Fig. 2A, d and e). BPA promoted a dose-dependent decrease

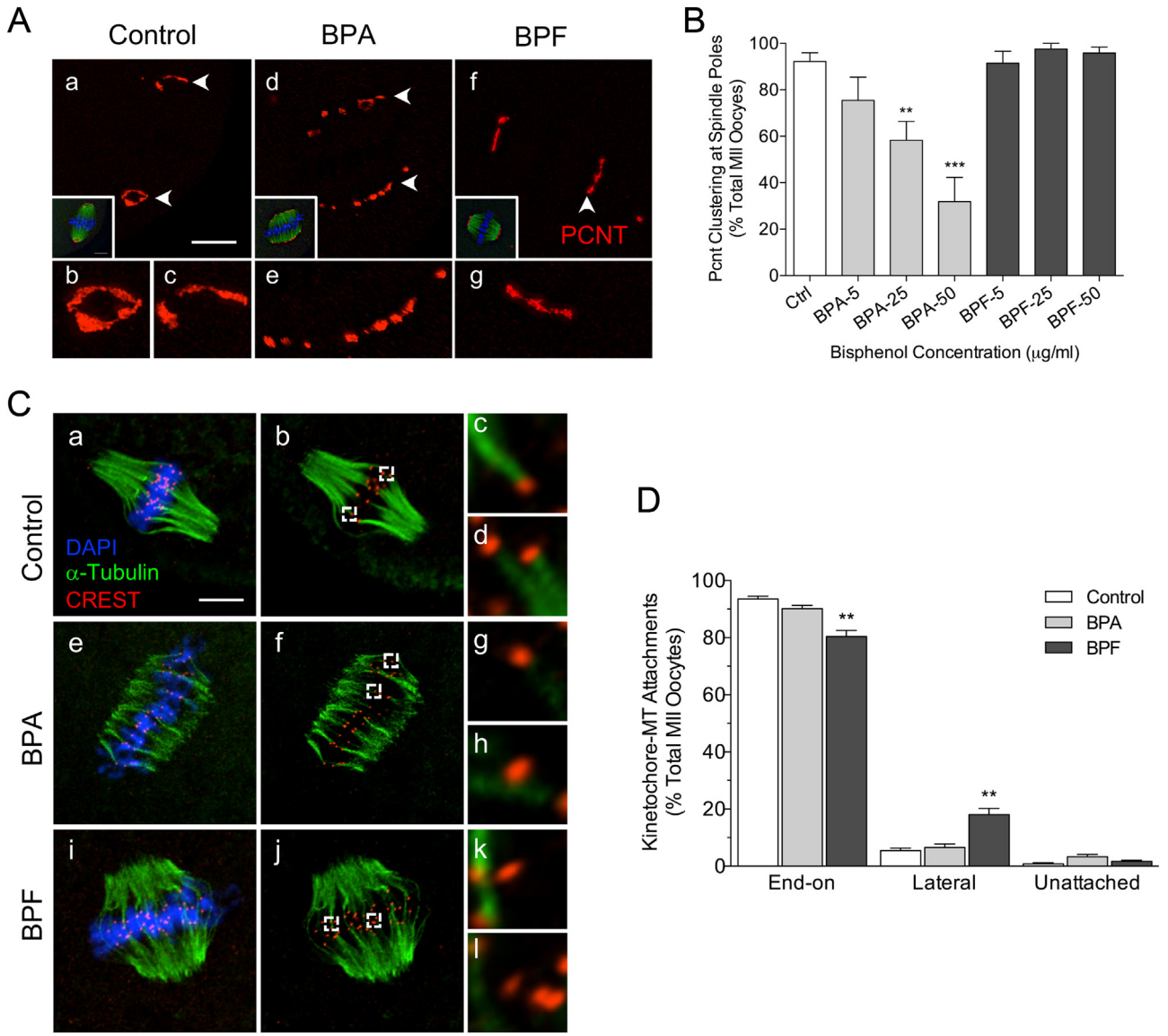

Figure 2 Bisphenol compounds disrupt PCNT clustering at meiotic spindle poles and chromosome-MT attachments at kinetochores. (A) Confocal imaging of PCNT distribution in ovulated control, BPA, and BPF-exposed oocytes following a 4 h culture. Oocytes were double-labeled with anti-PCNT (red, arrows) and anti-TPX2 (green). DNA is shown in blue. The spindle structure is shown in the insets. (B) The percent (mean ± S.E.M.) total oocytes with clustered PCNT at aMTOCs in a full or partial ring structure from three replicates (30–40 oocytes per group). (C) Confocal analysis of kinetochore-MT attachments and representative projection of a confocal Z-stacks showing chromosomes (blue), MTs (green), and kinetochores (red) labeled with anti-CREST in oocytes (20 per group) from the control, BPA, or BPF-treated groups. Maximal projections (a, e, and i). of optical sections (b, f, and j) showing normal end-on (c and d), weak end-on (g) and lateral kinetochore-MT (h and k) attachments as well as unattached kinetochores (l). Scale bar: 10 μm. (D) The percent (mean ± S.E.M.) of normal end-on and lateral kinetochore-MT attachments as well as unattached kinetochores assessed in control ($n = 20$), BPA ($n = 19$), and BPF ($n = 19$) exposed oocytes. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the control group.

in the percentage of oocytes with clustered PCNT at the spindle poles (Fig. 2B). Meiotic spindles with broad poles in BPF-treated oocytes also showed partial fragmentation of PCNT, but to a lesser extent than BPA exposure and did not reach statistical significance (Fig. 2B). These data demonstrate that exposure to bisphenol disrupts PCNT clustering at the meiotic spindle poles, which may impair aMTOC integrity and/or function.

Considering the significant spindle disruption, we determined whether a short exposure to BPA or BPF (50 µg/mL) perturbs chromosome-MT attachments in ovulated oocytes. Analysis of kinetochore–MT attachments after the 4 h culture (Fig. 2C) revealed a lower incidence of normal end-on attachments and increased erroneous lateral attachments in oocytes exposed to BPF (Fig. 2D). Interestingly, BPA exposure did not significantly alter the incidence of end-on attachments, despite promoting a more pronounced disruption of the spindle organization. These data demonstrate that even a brief BPF exposure promotes erroneous kinetochore–MT interactions in oocytes and also indicate that different bisphenol compounds may exert distinct detrimental effects on MTs.

Bisphenols promote a rapid onset of MII spindle disruption that is reversible upon compound removal

Live-cell imaging was used to assess the dynamics of spindle and chromosome configurations in real-time during the 4 h BPA or BPF (50 µg/mL) exposure (Fig. 3 and Video 1). Control MII oocytes retained a normal bipolar spindle organization during culture, with less than 15% of oocytes showing broader poles after 4 h. In contrast, BPA promoted the rapid onset (~15–30 min) of spindle disruption in 78% of oocytes (Fig. 3B and C). This was characterized by progressive shortening of the spindle MTs and pronounced widening of the spindle structure, with no discernable MT focusing toward the poles. The chromosomes remained aligned in an unusually elongated metaphase plate-like formation. Exposure to

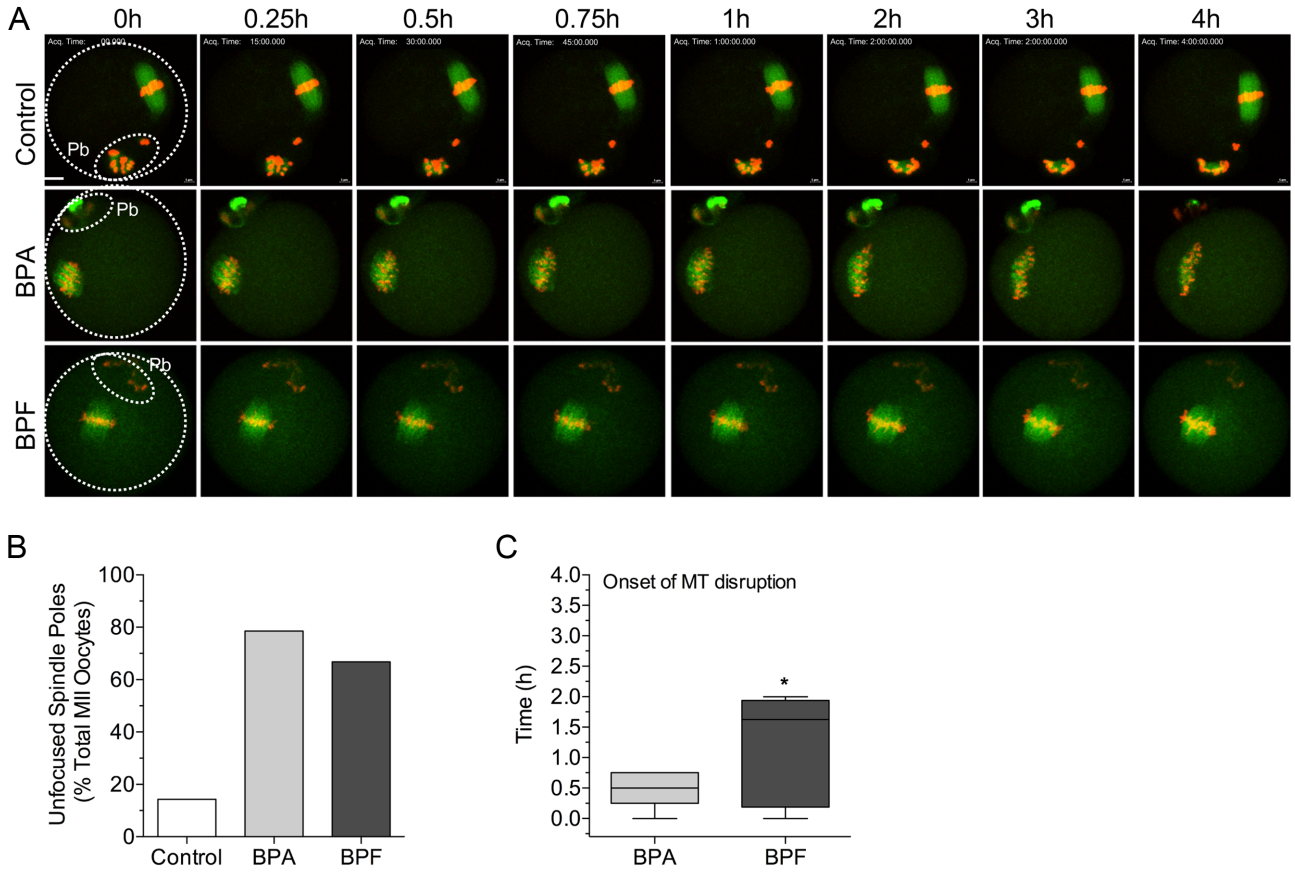

Figure 3 Live cell imaging reveals rapid onset of meiotic spindle disruption in response to bisphenol exposure. (A) Confocal time-lapse images (maximum intensity z-projections at 5 µm intervals with a 100 µm range) at 15 min intervals, showing spindle and chromosome configurations in MII oocytes during a 4 h incubation in media supplemented with 50 µg/mL of either BPA ($n = 14$) or BPF ($n = 18$). Control ($n = 14$) oocytes were cultured in media alone. Oocytes express H2B-RFP and MAP4-EGFP to label the chromosomes (red) and MTs (green), respectively. Pb: polar body. Scale bar: 10 µm. (B) Percent of total oocytes from three replicates that show MT disruption in control, BPA, or the BPF-treated group. (C) Onset (h) of MT disruption in BPA and BPF-treated oocytes. There was an initial imaging delay of ~10–15 min from the time the oocytes were transferred into the environmental chamber, due to confocal/imaging setup. *$P < 0.05$ between groups.

BPF also disrupted meiotic spindle organization in 67% of the oocytes, although less pronounced relative to BPA. Due to a brief 10–15 min interval between oocyte transfer to BPA or BPF-supplemented media and confocal imaging set up, broader spindle poles were already evident in the initial oocyte images, underscoring to the rapid effect of these compounds on MTs. These data support that BPA and BPF promote rapid and sustained meiotic spindle disruption, indicative of altered MT polymerization and/or depolymerization dynamics.

Video 1
Time-lapse imaging of oocytes during bisphenol exposure. Metaphase II (MII) oocytes with an assembled meiotic spindle were cultured for 4 h in media alone (top, control) or media supplemented with 50 mg/mL of BPA (middle) or BPF (bottom). Time-lapse (15 min intervals) movie of laser scanning confocal Z-stack reconstruction (every 5 µm) of oocytes expressing H2B-RFP (red) and MAP-4-EGFP (green) that label chromosomes and spindle microtubules, respectively. This video (http://movie-usa.glencoesoftware.com/video/10.1530/REP-19-0494/video-1) is available from the online version of the article at https://doi.org/10.1530/REP-19-0494.

Next, we tested whether the bisphenol-mediated spindle disruption can be reversed upon compound withdrawal (Fig. 4). Ovulated oocytes were cultured with BPA or BPF (25, 50 µg/mL), then thoroughly washed three times, and cultured for an additional 4 h in media alone (Fig. 4A and B). Quantitative analysis confirmed that the spindle length and pole width were corrected with bisphenol removal (Fig. 4B, C and D). In control oocytes there was no difference in spindle length or spindle pole width between the 4 h and 8 h culture times. However, the spindle length (Fig. 4C) increased significantly and the pole width (Fig. 4D) decreased when BPA or BPF-exposed oocytes were washed and transferred to media alone. Therefore, the ratio of pole width to spindle leng (Fig. 4E) in each fresh media group was similar to the controls. These data demonstrate that the rapid and pronounced effect of bisphenols on MT dynamics is reversible, at least after a brief 4 h exposure time. Moreover, it further underscores that BPA and BPF likely disrupt the regulation of MT polymerization/depolymerization, pointing to a possible mechanism of action.

BPA and BPF disrupt MT stability as well as kinesin motor protein (HSET/KIFC1) along the MTs

To assess potential mechanisms of spindle disruption, we tested the effect of BPA and BPF (50 µg/mL) on spindle MT stability as well as the dynamics of MT regrowth following depolymerization induced by cold treatment (Fig. 5A, B and C). After incubation at 4˚C for 30 min, cold-stable MTs were detected mainly in proximity to the spindle poles in control oocytes (Fig. 5A). Notably, the BPA-exposed oocytes showed almost no cold stable spindle MTs, compared to the control and BPF-treated oocytes (Fig. 5A and B). Moreover, after a brief (5 min) incubation at 37˚C in pre-warmed media, MT regrowth and typical bipolar spindle assembly was observed in control oocytes. In marked contrast, the MT organization was highly disrupted in the BPA-group with the majority of reforming spindles characterized by unfocused (29%) or multipolar (62%) structures (Fig. 5A and C). Surprisingly, the effects of BPF during cold treatment differed from BPA, as cold stable MTs were detected in the majority of BPF-treated oocytes after 30 min cold treatment and persisted even after an extended 1 h culture at 4˚C (Fig. 5D and E). Most BPF-treated oocytes contained multiple cold stable MTs after 1 h cold treatment, while control oocytes showed few to no cold stable MTs (Fig. 5D and E). The reforming spindles in BPF-treated oocytes were shorter with a small group (20%) showing unfocused poles, but showed no multipolar defects. These data establish that BPA promotes a significant decrease in oocyte MT stability and are highly disruptive to spindle organization upon MT regrowth. In contrast, cold-stable MTs persist in BPF-treated even after prolonged cold exposure, pointing to differences in BPA and BPF action on MTs.

We also examined whether BPA or BPF exposure disrupts kinesin-14 (HSET/KIFC1) localization along the spindle microtubules. This MT-associated minus-end motor protein plays an important role in MT crosslinking/bundling during spindle assembly and its inhibition leads to shorter spindle lengths. Moreover, it plays an important role in spindle pole focusing (Cai *et al.* 2009). High resolution SR-SIM analysis showed HSET labeling along the meiotic spindle microtubules in control as well as BPA- and BPF treated oocytes (Fig. 5F). Interestingly, fluorescence analysis revealed lower HSET labeling along MTs in BPA-treated oocytes, while it was seemingly higher in the BPF-treated group (Fig. 5G). These data indicate that BPA-mediated spindle disruption and decreased MT stability may be associated with reduced kinesin-14 motor protein. In contrast, BPF-exposed oocytes show sustained MT stability and HSET labeling along the MTs.

Inhibition of Aurora Kinase A activity limits bisphenol-mediated spindle pole disruption

To gain insight into possible BPA or BPF targets that disrupt MTs, we explored a potential interaction with the Aurora Kinase A (AURKA) pathway, which plays an important role in spindle assembly and MT nucleation (Sardon *et al.* 2008, Solc *et al.* 2012, Bury *et al.* 2017). We assessed the spindle organization in ovulated oocytes (Fig. 6) in response to BPA and BPF exposure, with or without a selective AURKA inhibitor (MLN8237). The inhibitor decreased phosphorylated (activate) pT288

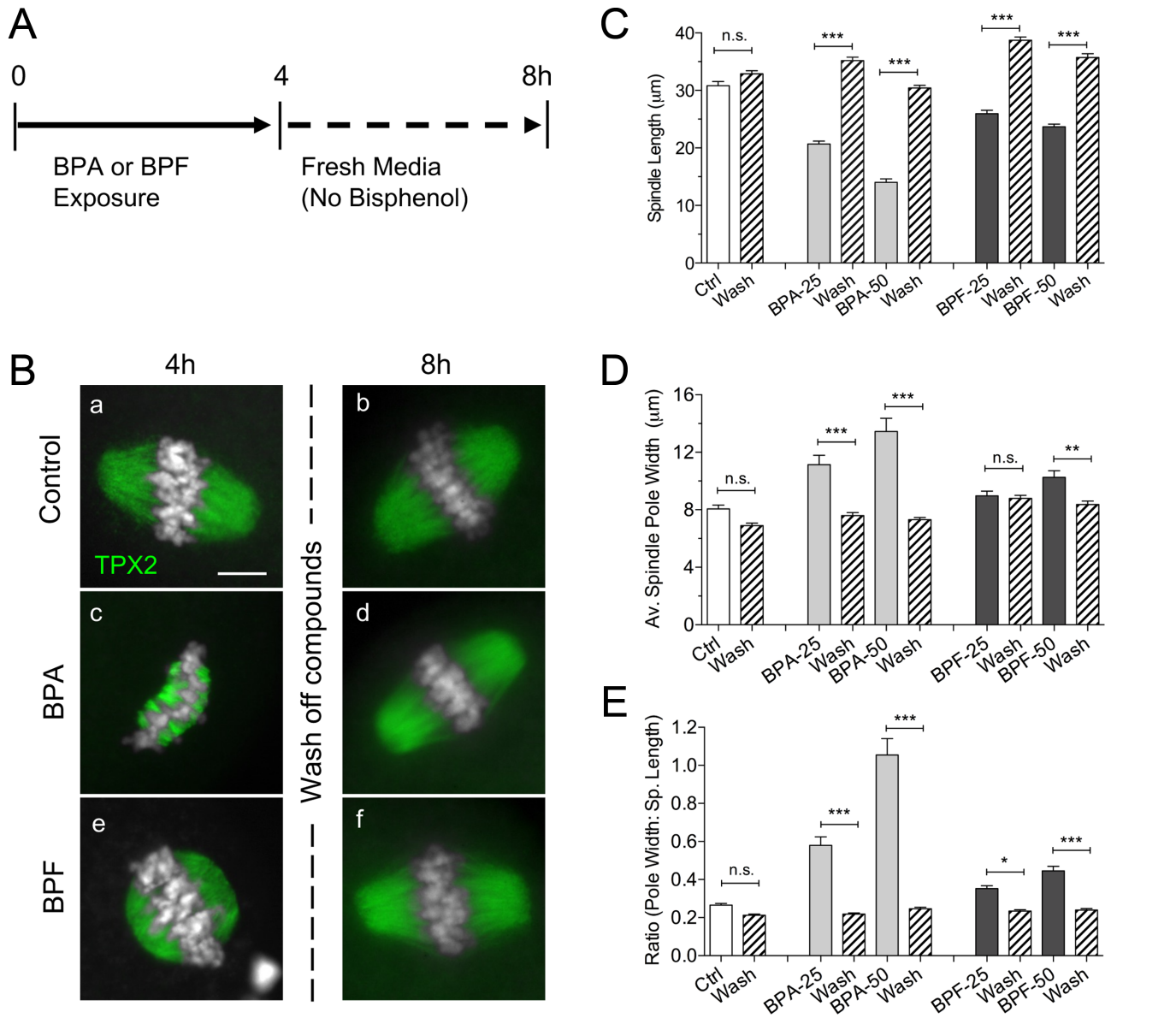

Figure 4 Bisphenol-mediated disruption of the meiotic spindle is reversible after compound removal. (A) Schematic outline of culture duration to assess spindle structure in ovulated oocytes following BPA or BPF exposure and subsequent transfer and incubation in fresh media alone. (B) Representative fluorescence microscopy images of oocytes after 4 h culture with BPA or BPF (a, c, and e) and following an additional 4 h in bisphenol-free media (b, d, and f) at 8 h. MTs are shown in green and chromosomes in grey. Scale bar: 10 μm. Quantitative analysis (mean ± S.E.M.) of the (C) meiotic spindle length (pole-to-pole distance), (D) average pole width, and (E) ratio of the pole width to spindle length measured in oocytes (50–80 total per group), from three replicates at 4 h post bisphenol exposure (solid bars) compared to bisphenol removal and culture (hatched bars). *$P<0.05$, **$P<0.01$, and ***$P<0.001$ differences between bisphenol-exposure (4 h) and compound removal (8 h, Wash). n.s.: no statistical difference between groups.

AURKA levels in oocyte lysates, while total AURKA was unchanged. No differences in pT288 AURKA or total AURKA were detected in response to BPA or BPF alone (Fig. 6A and B) and pT288AURKA was detected at the poles in all groups (Fig. 6C). Inhibition of AURKA in control oocytes promoted chromosome misalignment and lead to a significant decrease in the spindle length as well as spindle pole width. Moreover, pT288AURKA was detected in small foci at the highly focused poles instead of the typical ring-like configuration (Fig. 6C). Incubation with BPA or BPF, together with the AURKA inhibitor, further reduced the spindle length (Fig. 6D and F). However, the spindle poles remained focused in both BPF and PBA-treated oocytes when the AURKA inhibitor was present (Fig. 6E and G). These data identify an important link between the bisphenol-mediated spindle MT disruption and AURKA activity, suggesting that BPA

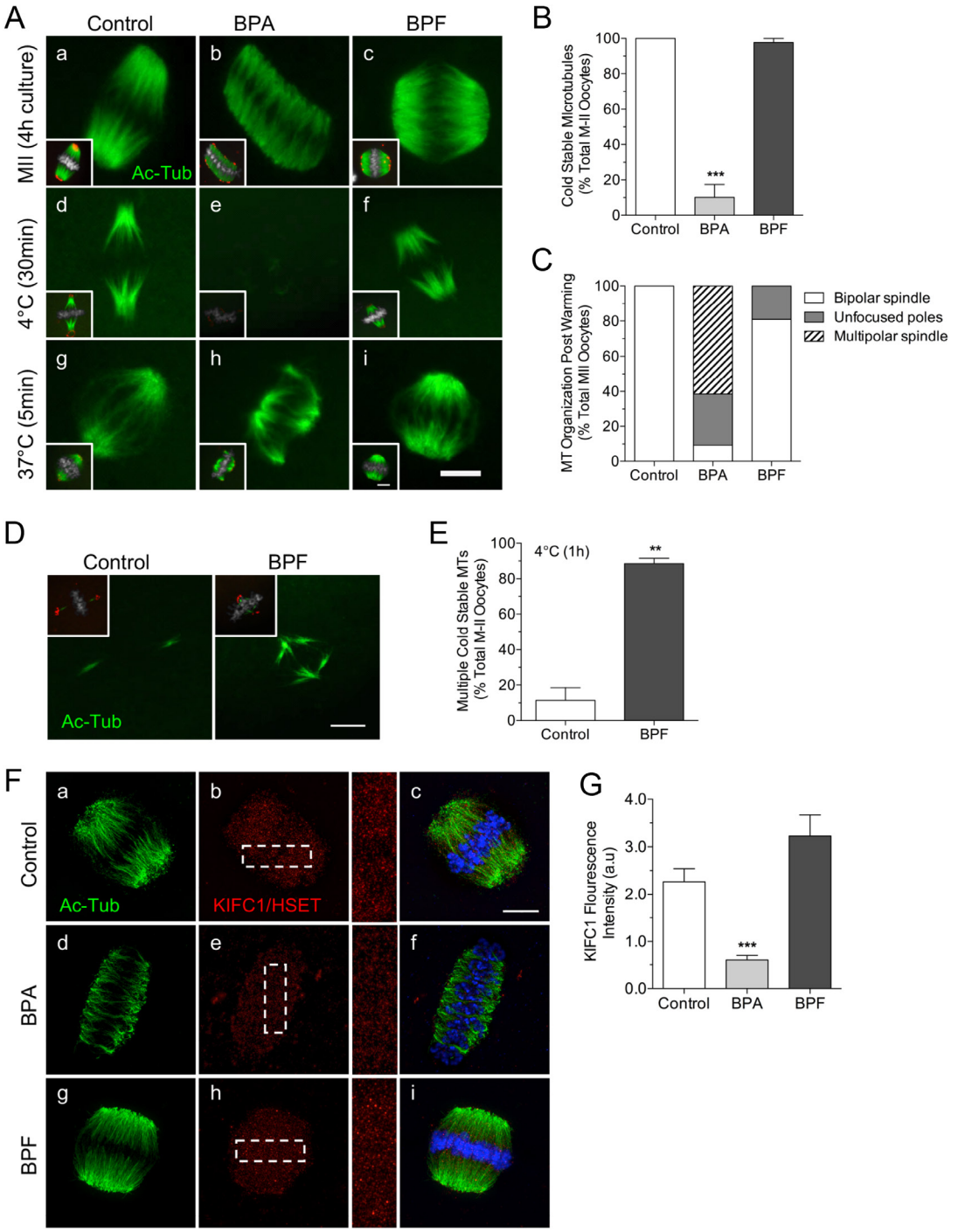

Figure 5 Exposure to BPA disrupts spindle MT stability and Kinesin-14 (HSET/KIFC1) labeling along the MTs. (A) Oocytes from control ($n = 197$), BPA ($n = 233$), or BPF ($n = 203$) treatment (50 µg/mL) groups. Ovulated oocytes were fixed after 4 h culture (a, b, and c), 30 min post cold-treatment at 4°C to depolymerize MTs (d, e, and f) and 5 min rewarming at 37°C to assess MT regrowth (g, h, and i) using fluorescence microscopy. Insets show spindle structure with chromosomes. All oocytes were double labeled with anti-acetylated tubulin (Ac-Tub, green) and anti-PCNT (red). Chromosomes counterstained with DAPI (grey). (B) Percent (mean ± S.E.M.) total oocytes showing cold-stable MTs after 30 min at 4°C. (C) Spindle MT organization was assessed 5 min post rewarming at 37°C. Percent total oocytes containing an organized bipolar spindle, unfocused spindle poles or disorganized multi-polar spindle structures in the control, BPA, or BPF groups. (D) Control ($n = 73$) and BPF-treated ($n = 56$) oocytes following an extended 1 h incubation at 4°C. (E) Percent total oocytes with multiple cold-stable MTs after 1 h at 4°C. (F) High resolution (SR-SIM) microscopy analysis of HSET (red) labeling along the spindle MTs (Ac-Tub; green). A 4x magnification of the selected area (white box) for KIFC1/HSET (red) in each group. DAPI-labeled chromosomes shown in blue. Scale bar: 10 µm. (G) Relative fluorescence intensity of HSET between control and BPA or BPF-treated (50 µg/mL) ovulated oocytes (15 per group). $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the control group. a.u.: arbitrary units.

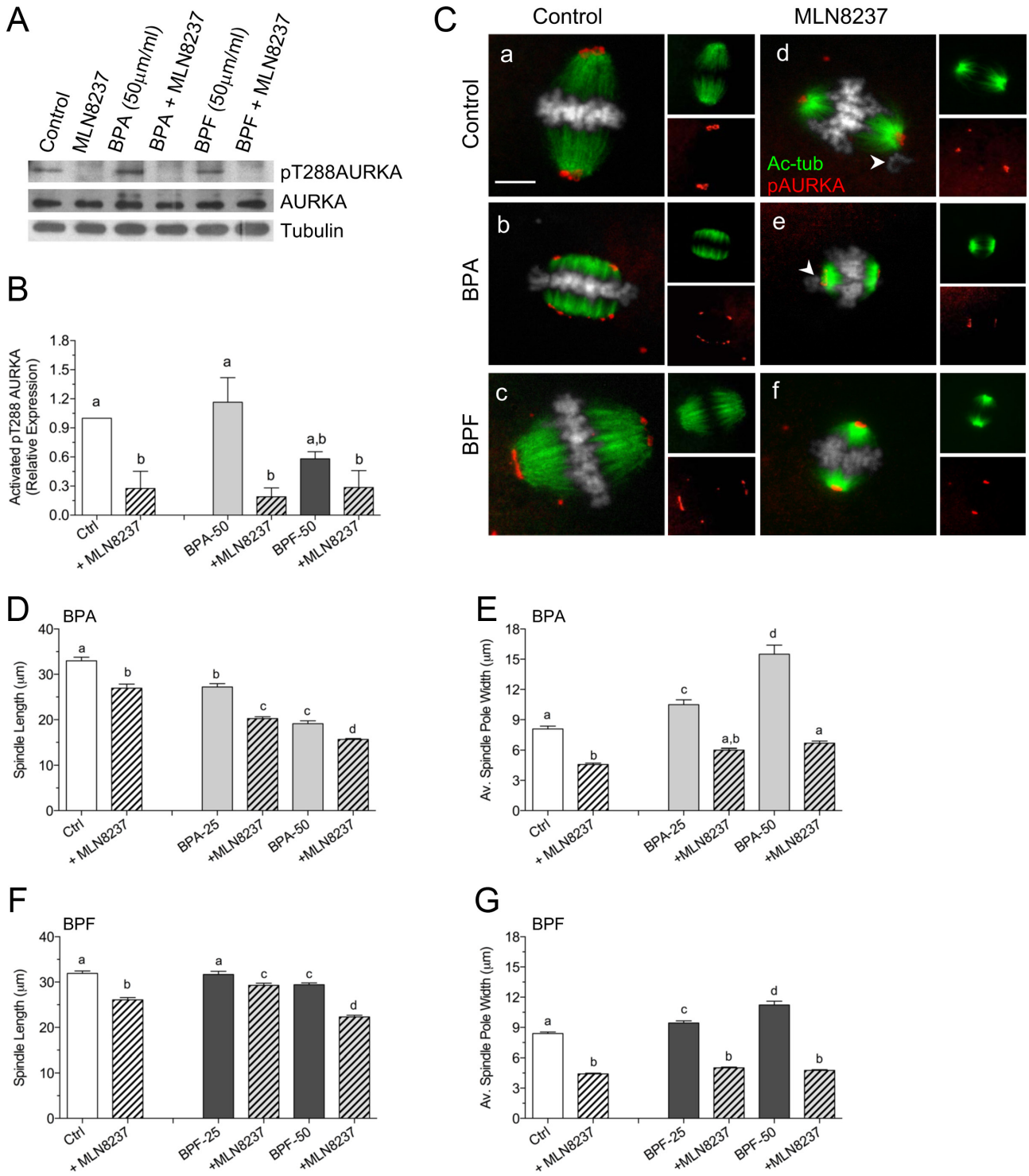

Figure 6 Inhibition of Aurora Kinase A (AURKA) limits bisphenol-mediated spindle pole disruption. (A) Western blot (five replicates) of phosphorylated/active AURKA (pT288AURKA), total AURKA and control β-tubulin protein levels in control, BPA, or BPF-treated oocytes lysates (50 oocytes/lane) either alone or together with MLN8337. (B) The expression levels of active pT288AURKA compared between treatment groups and the control (media alone), which was normalized to 1.0. (C) Representative fluorescence microscopy images of oocytes from control as well as BPA or BPF treatment (50 μg/mL) groups following a 4 h culture with or without a specific AURKA inhibitor, MLN8237 (500 nM). Oocytes were double labeled with anti-acetylated tubulin (green) and pT288 AURKA (red). Chromosomes (grey) were counterstained with DAPI. Scale bar: 10 μm. Quantitative analysis (mean ± S.E.M.) of the (D and F) meiotic spindle length (pole-to-pole distance) and (E, G) average spindle pole width assessed in BPA or BPF-treated oocytes (50–80 total oocytes per group, from three replicates) – either alone (solid bars) or together with MLN8237 (hatched bars). Control oocytes were cultured in either media alone or with MLN8237. Different letters denote statistical significance at $P < 0.05$.

and BPF may directly/indirectly disrupt AURKA action that regulates meiotic spindle pole focusing.

Discussion

The current study tested the effects and underlying mechanisms of bisphenol action on assembled meiotic spindle organization in ovulated oocytes. Our data reveal that both BPA and BPF promote rapid and striking MT disruption, which significantly impairs meiotic spindle organization and stability. Notably, we identify an important link between these MT defects and altered distribution of key spindle associated factors, PCNT and kinesin-14 motor protein (HSET/KIFC1), as well as Aurora Kinase A activity. Surprisingly, BPA and BPF show distinct effects on these mechanisms raising concerns regarding the use of BPF as a 'safe' substitute. These data provide new mechanistic insight into the detrimental effects of two common bisphenol compounds on oocyte spindle stability, which has important implications for fertilization and subsequent embryo development.

Ovulated oocytes were used to test BPA and BPF action, specifically on the fully assembled meiotic spindle, during acute exposure. The test bisphenol concentrations (5–50 µg/mL) used are similar to previous studies with human (Machtinger *et al*. 2013) and mouse (Nakano *et al*. 2016) oocytes that limit oocyte maturation to MII, but are significantly higher than total or free BPA levels detected in human urine that are typically in the ng/mL range (Teeguarden *et al*. 2011, Liao & Kannan 2012). Our aim was to use an acute (4 h), high dose exposure to address the underlying mechanisms of bisphenol action. Brief (4 h) exposure to either bisphenol compound leads to rapid disruption of spindle organization, denoted by a dose-dependent decrease in spindle length together with a striking increase in pole width. Higher concentrations of BPA resulted in remarkably short spindles with highly unfocused poles, a subset of which exhibit splayed MTs. BPF promotes similar alterations in spindle structure and, although less pronounced than BPA, this damaging effect adds to the growing safety concerns regarding its use as a common BPA replacement analog (Sartain & Hunt 2016). Disrupted spindle organization has been observed during mitotic division in somatic cells (Pfeiffer *et al*. 1997) as well as mammalian oocytes cultured with bisphenol compounds during meiotic maturation (Can *et al*. 2005, Eichenlaub-Ritter *et al*. 2008, Machtinger *et al*. 2013, Zalmanova *et al*. 2017, Campen *et al*. 2018). However, bisphenol exposure during oocyte maturation precluded differentiating between specific effects on spindle formation/organization and meiotic arrest, as a high percentage of bisphenol-treated oocytes fail to progress to MII (Can *et al*. 2005, Eichenlaub-Ritter *et al*. 2008, Machtinger *et al*. 2013). Our analyses support that BPA and BPF directly disrupt the meiotic spindle. Interestingly, the chromosomes remain congressed in an abnormally elongated metaphase-like configuration in BPA and BPF-treated oocytes. Yet, analysis of kinetochore-MT attachments reveals fewer normal bioriented end-on attachments, and increased erroneous lateral MT attachments in BPF-exposed oocytes. Studies indicate that lateral chromosome-MT attachments are not resolved during MII-arrest in oocytes (Kouznetsova *et al*. 2019) and do not satisfy the spindle assembly checkpoint (Kuhn & Dumont 2017). Longer exposure to either BPA or BPF conceivably exacerbates the incidence of erroneous chromosome-MT interactions and likely accounts for the significant chromosome misalignment and aneuploidy observed in oocytes exposed to bisphenols during meiotic maturation (Hunt *et al*. 2003, Can *et al*. 2005, Machtinger *et al*. 2013). Recent studies in somatic cells also support that BPA disrupts spindle-kinetochore attachments (Kim *et al*. 2019). Moreover, our data are indicative of disrupted spindle MT dynamics. Notably, live cell imaging confirmed the rapid onset of MT disruption, which can be reversed upon compound withdrawal – at least after a short exposure time. This rapid and reversible effect underscores that BPA and BPF disrupt the regulation of MT dynamics (polymerization/de-polymerization) and organization, pointing to a possible mechanism of action.

Assessment of spindle MT stability following cold treatment reveals damaging effects of bisphenols. Specifically, we demonstrate that BPA promotes the loss of cold stable MTs in oocytes, indicating reduced MT stability and/or disruption of MT dynamics that likely contribute to the shorter spindle lengths. This is consistent with previous studies that identified tubulin as a BPA target protein (George *et al*. 2008) and demonstrated reduced MT assembly in somatic cells exposed to bisphenols (Pfeiffer *et al*. 1997). BPA also promotes MT disassembly in plant cells, disrupting both interphase and spindle MTs (Adamakis *et al*. 2019). In addition, our data reveal that spindle MT assembly and organization are significantly impaired upon rewarming following cold treatment, leading to the formation of unfocused and aberrant multipolar structures. This is presumably linked to the BPA-mediated fragmentation of PCNT, which can establish multiple sites of MT nucleation. Multipolar spindles have also been observed in somatic cells and fertilized sea urchin eggs exposed to BPA (George *et al*. 2008). Surprisingly, multiple cold-stable MTs persisted in BPF-treated oocytes compared to control oocytes even after extended cold treatment, indicating that BPF also disrupts MT dynamics. Our data are consistent with previous studies that show similar damaging effects of BPS, another BPA replacement analog, on the meiotic spindle in oocytes including shorter spindles following exposure to low bisphenol levels during oocyte maturation and spindle assembly (Campen *et al*. 2018). Here, we provide novel evidence regarding the underlying mechanisms of action and potential differences between BPA and BPF action on

assembled spindle MTs. Further studies are needed to determine the underlying basis for this compound difference and to test whether long-term exposure to low bisphenol concentrations promotes similar disruptions in ovulated oocytes. Moreover, considering the important bi-directional communication between the oocyte and cumulus cells and bisphenol action on granulosa cells (Peretz *et al.* 2014, Mansur *et al.* 2017), it will be important to assess how the presence of surrounding cumulus cells may affect the oocyte response to BPA or BPF. Nevertheless, our analyses indicate that bisphenol-mediated disruption of MT dynamics likely contributes to the rapid shortening of the assembled meiotic spindle lengths and disrupts binding of spindle-associated factors.

To further explore possible mechanisms of bisphenol action, we assessed key spindle-associated proteins and demonstrated fragmented pericentrin (PCNT) distribution at the spindle poles and altered Kinesin-14 (HSET/KIFC1) labeling along the spindle microtubules. PCNT plays an essential role in spindle assembly and organization, functioning as a key scaffolding protein in somatic cell centrosomes (Zimmerman *et al.* 2004, Delaval & Doxsey 2010) and oocyte aMTOCs (Baumann *et al.* 2017), necessary for MT nucleation. In BPA-treated oocytes, PCNT was highly fragmented (dispersed) along the unfocused poles, contrasting with the normal clustered 'O' or 'C-like' configuration in control oocytes. Altered PCNT distribution seemingly mirrors spindle pole focusing with very broad poles showing greater PCNT fragmentation and BPF-treated oocytes showing limited disruption of the protein. Similarly, BPA exposure in somatic cells leads to multiple MTOCs (Ochi 1999) and perturbs PCNT distribution in oocytes during meiotic maturation (Can *et al.* 2005). Moreover, oocyte-conditional knockdown of PCNT leads to significantly shorter MII spindles with broader poles (Baumann *et al.* 2017), supporting its role in MT formation and focusing of meiotic spindle poles. Thus, bisphenol-mediated alterations in oocyte PCNT distribution likely affect aMTOC integrity and/or function, contributing to the meiotic spindle defects, including multipolar spindle formation following cold treatment and MT regrowth. Further studies are needed to determine the underlying mechanisms by which bisphenols promote PCNT fragmentation at the spindle poles. Assessment of key MT-associated proteins showed no overt differences in TPX2, known to promote MT nucleation and spindle organization (Brunet *et al.* 2008, Helmke & Heald 2014). A recent study report disrupted TPX2 in somatic cells exposed to BPA (Kim *et al.* 2019). Whether longer exposure to bisphenols alters TPX2 distribution at the oocyte spindle remains to be determined. Notably, BPA-treated oocytes show reduced labeling of Kinesin-14 (HSET/ KIFC1) along the microtubules while it persisted after BPF exposure. This MT-associated minus-end motor protein plays an important role in MT crosslinking during spindle assembly, regulating spindle length and pole organization. Studies in yeast and somatic cells demonstrated that HSET inhibition leads to shorter spindle lengths (Cai *et al.* 2009, Hepperla *et al.* 2014). In mouse oocytes, this kinesin also plays a key role in meiotic spindle formation and pole focusing, with suppression of HSET resulting in broader meiotic spindle poles and overexpression promoting elongated and focused spindles (Mountain *et al.* 1999, Bennabi *et al.* 2018). In sum, our data point to potential differences in BPA and BPF action on spindle MTs and support that BPA-mediated spindle disruption is associated with altered distribution of key aMTOC and MT-associated factors that regulate spindle architecture.

Furthermore, we identified an important link between the bisphenol-mediated spindle pole disruption and Aurora kinase A (AURKA) activity. This serine/threonine kinase plays an essential role in both mitotic (Sardon *et al.* 2008) as well as meiotic (Solc *et al.* 2012, Bury *et al.* 2017) spindle assembly and organization. While mouse oocytes express three isoforms, Aurora kinase -A, -B and -C (Nguyen & Schindler 2017), AURKA specifically localizes to aMTOCs and contributes to rapid microtubule growth during meiotic spindle formation (Bury *et al.* 2017). Hence, spindle length was not rescued by inhibition of AURKA. In fact, the AURKA inhibitor promotes shorter spindles and misaligned chromosomes, consistent with previous studies (Solc *et al.* 2012, Bury *et al.* 2017). Notably, however, we demonstrate that reducing AURKA during BPA or BPF exposure significantly limited bisphenol-mediated spindle pole broadening such that the spindle poles remained focused, revealing a potential interaction. Neither BPA nor BPF exposure altered the total AURKA or activated pT288AURKA levels. Moreover, pT288AURKA localizes to the disrupted spindle poles, indicating that the bisphenol compounds potentially affect the localization and/or activity of down-stream targets of AURKA. Key factors that regulate pole organization and spindle architecture, such as NuMA and MCAK, are phosphorylated by AURKA (Zhang *et al.* 2008, Gallini *et al.* 2016, Zong *et al.* 2016). Our data identify an important link between the bisphenol-mediated spindle MT disruption and AURKA activity, which may affect key spindle-associated factors.

In summary, our findings provide novel insight regarding the direct effect(s) of acute bisphenol exposure on assembled meiotic spindle organization as well as the mechanisms of action. We demonstrate rapid MT-disrupting activity by both BPA and BPF that is highly detrimental to oocyte spindle organization and stability, which may increase the risk of chromosome segregation errors and compromise subsequent embryo development upon fertilization. These defects are associated with the disruption of MT stability and key spindle associated factors (PCNT, KIFC1/HSET). Moreover, we reveal differences in BPA and BPF action

and identify an important link between bisphenol-mediated spindle disruption and AURKA activity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

L Y performed experiments, analyzed data, and co-wrote the manuscript. C B performed experiments. R D L F contributed to experiment design and manuscript preparation and provided reagents. M M V conceived and designed experiments, analyzed data, wrote the manuscript, and supervised the research.

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