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Bisphenol A in culture media and plastic consumables used for ART

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STUDY QUESTION: Do the embryo culture media and plastic materials used during assisted reproductive technology (ART) laboratory procedures expose embryos to bisphenol A (BPA)?

SUMMARY ANSWER: BPA was not detected in embryo culture media or protein supplements at concentrations above those encountered in normal patient serum and follicular fluids.

WHAT IS KNOWN ALREADY: BPA is strongly suspected of altering the epigenome during mammalian development. Medical devices have been shown to be a source of BPA exposure in adult and neonatal intensive care units.

STUDY DESIGN, SIZE, DURATION: An analytical study of ART culture media and plastic labware products was performed under conditions close to routine practice and if BPA was detected, tests were carried out under more stringent conditions.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Two single-step embryo culture media, two sequential media and three different protein supplements [a purified human serum albumin (HSA), a synthetic serum substitute, and a recombinant HSA] were tested for BPA. Thirty-three different plastic consumables, used from oocyte collection through to embryo transfer, were tested for their ability to leach BPA into their surrounding environment.

BPA concentrations were measured according to a previously described liquid chromatography/mass spectrometry method. This method is linear over the calibration range from 0.5 to 100 ng/ml using a linear model weighted by $1/X^2$ and validated in terms of selectivity, linearity, repeatability, reproducibility and limit of quantification (0.5 ng/ml).

MAIN RESULTS AND THE ROLE OF CHANCE: Neither the culture media nor the protein supplements were shown to contain detectable levels of BPA. None of the plastic materials leached BPA into the surrounding medium at levels higher than the upper limit detected previously in serum and follicular fluids in women (about 2 ng/ml). However, the plastic of the three tested strippers used for oocyte denudation/embryo handling did contain BPA. Two of these strippers are made with polycarbonate, a plastic whose synthesis is known to require BPA.

LIMITATIONS, REASONS FOR CAUTION: This study is limited to the ART media and materials tested here and using a BPA assay with a limit of quantification at 0.5 ng/ml. A minimum volume was required for testing, and one type of plastic labware could not be tested in conditions identical to those in routine use.

WIDER IMPLICATIONS OF THE FINDINGS: Although we demonstrated that some plastic materials used in ART contain BPA, under routine conditions none appear capable of leaching BPA at levels higher than those from maternal internal exposure. However, BPA is strongly suspected of altering the epigenome. Since important epigenetic modifications occur in the early embryonic stage, it is questionable whether plastics that contain BPA, polycarbonate in particular, should be used in the manufacture of plastic consumables for ART procedures.

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Key words: bisphenol A / assisted reproductive technology / plastic consumables / environmental toxicants / epigenetic

Introduction

Bisphenol A (BPA) is a chemical in widespread use that is present in a variety of common consumer products, leading to two main exposure routes, food and non-food sources (Geens *et al.*, 2012). Detected in more than 90% of the US general population (Calafat *et al.*, 2008), BPA is notably present in polycarbonate plastics, in the epoxy resin liners of aluminum cans, and in thermal paper (WHO, 2011). BPA acts as an endocrine-disrupting chemical through physiological receptors such as genomic estrogen receptors (ER) 1 and 2, membrane-bound ERs, androgen receptors, peroxisome proliferator-activated receptor gamma, and thyroid hormone receptors (reviewed in Richter *et al.*, 2007).

The concerns regarding the effects of BPA exposure on reproductive health outcomes have led to numerous publications based on *in vitro*, animal and human studies. Recent reviews on this topic (Caserta *et al.*, 2014; Peretz *et al.*, 2014) have emphasized that targets of BPA include gametes (meiosis, folliculogenesis, steroidogenesis) and the uterus (endometrial proliferation, receptivity). In humans, however, knowledge of the role of BPA as a reproductive toxicant is still sparse. An epidemiological study failed to demonstrate an effect of BPA on spontaneous fertility in humans (Buck Louis *et al.*, 2014). However, after controlled ovarian stimulation (COS), the level of urinary BPA (range <0.4–25.5 μg/l; mean ± SD 2.52 ± 3.2 μg/l) was inversely correlated with the number of oocytes retrieved. This led, for each log unit increase in urinary BPA, to an average decrease of 12% in the number of oocytes retrieved and an average decrease of 213 pg/ml in peak serum estradiol on the day of ovulation induction (Mok-Lin *et al.*, 2010). Moreover, in women, urinary BPA (range <0.4–26.5 μg/l, mean 2.32 μg/l) is inversely correlated with the number of mature oocytes after COS (Ehrlich *et al.*, 2012a). Antral follicle count, a marker of ovarian reserve and a good predictor of ovarian response to COS, has been shown to be significantly decreased with increasing quartiles of urinary BPA (range <0.4–20.5 μg/l; mean ± SD 1.6 ± 2.0 μg/l, Souter *et al.*, 2013). Regarding oocyte maturation (Machtinger *et al.*, 2013), a negative dose–response correlation has been observed between human oocyte maturation rate *in vitro*, the proportion of bipolar spindles in metaphase II oocytes and the concentration of BPA added to the maturation medium (ranging from 20 ng/ml to 20 μg/ml, whereas human follicular fluid BPA concentration is known to be 1–2 ng/ml, Ikezuki *et al.*, 2002). Finally, there were increased odds of implantation failure with higher quartiles of urinary BPA concentrations (ranging from <0.4 to 26.5 μg/l; mean ± SD 1.53 ± 2.22, Ehrlich *et al.*, 2012b). However, it is not known if these failures were of uterine and/or embryonic origin.

In vitro exposure of mouse embryos to an environmentally relevant dose of BPA (1 nM, or 0.228 ng/ml) from the 2-cell stage to the blastocyst stage had no effect on litter size or birthweight when these blastocysts were transferred to untreated foster mothers, but increased the weaning weight of pups (post-natal day 21) by 39% compared with controls (Takai *et al.*, 2001). Furthermore, another study exposing pregnant mice to BPA at an environmentally relevant dose during the post-implantation period (from 11 to 17 days post-coitus) showed that pups treated with BPA *in utero* were significantly heavier than controls at weaning on post-natal day 22, despite having a similar body weight at birth (Howdeshell *et al.*, 1999). Furthermore, it has been shown recently in the mouse that maternal exposure, during the preimplantation period only, to 20 μg/kg body weight/day of oral BPA (below the US Food and Drug Administration acceptable daily intake of 50 mg/kg body weight/day) delays testes development and reduces testosterone production due to deficits in the histone acetylation of the StaR gene (Hong *et al.*, 2016). This clearly demonstrates that the preimplantation period should be scrutinized as deeply as the post-implantation period when evaluating the effects of prenatal exposure to an endocrine disruptor chemical such as BPA.

In this regard, since plastic-containing medical devices have been shown to be a source of BPA exposure in adult and neonatal intensive care units (Calafat *et al.*, 2009; Duty *et al.*, 2013; Huygh *et al.*, 2015), it is important to determine whether the manipulations of gametes and embryos performed during an assisted reproductive technology (ART) procedure could expose them to external chemical contaminants such as BPA.

Materials and Methods

Since the aim of this study was to determine whether BPA is released by plastic consumables used in ART laboratories, neither gametes nor embryos were used and ethics committee approval was unnecessary.

Media and protein supplements

Four different human embryo culture media were tested both in their commercial containers and under routinely used conditions (50 μl microdrops under oil after incubation at 37°C, 6% CO_2 for 24, 48 or 96 h in a normal 4-well IVF dish, Nunc®, catalog number 179830). Two single-step (Global, LifeGlobal®, USA; CSC, Irvine Scientific®, USA) and two sequential media (G-1™ v5 PLUS/ G-2™ v5 PLUS, Vitrolife®, Sweden; ISM1/BlastAssist, Origio®, Denmark) were investigated. Two different batches of each medium were evaluated in triplicate.

Three different protein supplements were also tested: purified human serum albumin (HSA®, LifeGlobal®, USA) added to Global medium at 5 or 10%; a synthetic serum substitute (Serum Substitute Supplement, Irvine Scientific®, USA) added to CSC medium at 5 or 10%; recombinant HSA (G-MM®, Vitrolife, Sweden) added at 5% to Global medium. One batch of each protein supplement was tested in triplicate.

All samples were stored at −20°C in a Nunc® polypropylene 1.2 ml cryotube (ref. 055011, Dominique Dutscher, France). Stored volumes were 100 μl for media in their commercial containers and 45 μl for media in routine use conditions and for the protein-supplemented media. The cryotubes, the plastic tips used to sample and store the media and the oil used to overlay media microdrops had been previously excluded as they did not contain detectable BPA.

Plastic consumables

The plastic consumables tested were those used by six different French ART centers participating in a prospective observational study examining the relationships between women's BPA exposure and implantation success after ART (referenced as Clinical Trials number NCT02377219). Because the minimum volume of our BPA assay was 45 μl, the 33 different materials (detailed in Table I) were divided into two groups according to their internal volumes: large (≥45 μl) or small volume (<45 μl). The large volume group ($n = 23$) comprised two plastic syringes, two tubes, five aspiration needles and their connecting tubing for oocyte aspiration, seven plastic dishes for oocyte collection or embryo culture, five embryo transfer catheters and two syringes for embryo transfer. In this group, one batch of each plastic consumable was tested in triplicate (i.e. three different consumables) according to a two-step strategy. Firstly, the consumable was filled for 2 min with 37°C

Table I Details of the plastic labware analyzed in the study of bisphenol A (BPA) levels leached from materials used during assisted reproductive technology (ART) laboratory procedures.

Product type	No.	Brand	Origin	Name	Plastic[a]	Product reference
Plastic material with large internal volume (≥45 µl)						
Syringes for oocyte retrieval	1	BD	USA	BD Plastipak	PP[b,c], ER, SL	302188
	2	BD	USA	BD Plastipak Luer-Lok	PP[b,c], ER, SL	300912
Tubes for oocyte collection	3	Sarstedt	Germany	Tubes for collection	PP	62.515.028
	4	Corning	USA	Falcon tubes	PS	352001
Oocyte aspiration needles and connecting tubing	5	Vitrolife	Sweden	Follicle aspiration set	PTFE	14112
	6	Vitrolife	Sweden	Extension tube	PTFE	21085
	7	Origio	Denmark	Single lumen needle	PE	SL1703011
	8	Cook Medical	USA	Vacuum line	NA	G38692 K-DVLF-240
	9	Cook Medical	USA	Disposable extension tube	NA	G16329 J-DET-115000
Plastic dishes for embryo culture	10	Thermo Scientific	USA	Nunc IVF Petri dishes (untreated surface)	PS	150270
	11	Thermo Scientific	USA	Nunc IVF Petri dishes (Nunclon treated surface)	PS	150318
	12	Thermo Scientific	USA	Nunc IVF 4-well dishes (untreated surface)	PS	179830
	13	Thermo Scientific	USA	Nunc IVF 4-well dishes (Nunclon treated surface)	PS	144444
	14	Corning	USA	Falcon Easy-Grip dishes	PS	353004
	15	Corning	USA	Falcon IVF dishes	PS	353652
Plastic dishes for time-lapse embryo culture	16	Vitrolife/ FertiliTech	Sweden	EmbryoSlides	NA	FT-S-ES-D
Catheters for embryo transfer	17	Ellios Bio Tek	France	Elliocath	PE	NS1280545
	18	Ellios Bio Tek	France	Echocath	PU	NS1280555-E
	19	Laboratoire CCD	France	Frydman classic catheter 4.5	PE	1306045
	20	Laboratoire CCD	France	Frydman Memory standard model	NA	130MF45
	21	Cook Medical	USA	Guardia AccessET	NA	G53247
Syringes for embryo transfer	22	BD	USA	BD syringe Luer-Lok	PC[b], PP[c] SL	309628
	23	PentaFerte	Italy	1 ml Tube nude	PP	2022140
Plastic material with small internal volume (<45 µl)						
Syringe stoppers	24	B. Braun	Germany	Combi-Stoppers	PE	4495101
Pipette tips	25	VWR Collection	USA	Ultrafine tips	PP	732-0562
	26	Eppendorf	Germany	epTIPS Biopur	PP	30010035
	27	Thermo Scientific	USA	ART barrier tips	NA	2139-HR
	28	Sorenson BioScience	USA	LongReach pipette tips	PP	37650
	29	Gilson	USA	Pipetman Tips Diamond	PP	DF100ST
	30	Greiner Bio-One	Germany	Pipette tips	PP	685290
Strippers for oocyte denudation	31	Cook Medical	USA	Cook Flexipet pipette	PC	G26712K-FPIP-1140-10BS-5
	32	Origio	Denmark	Origio stripper	PC	MXL3-100
	33	Research Instruments	UK	EZ-Tip	NA	7-72-4135/20

ER, epoxy resin; PC, polycarbonate; PE, polyethylene; PP, polypropylene; PS, polystyrene; PTFE, polytetrafluoroethylene (Teflon); PU, polyurethane; SL, silicone lubricant; NA, not available.
[a]As specified in the manufacturer's website or documentation.
[b]Body of the syringe.
[c]Piston of the syringe.

pre-warmed G-MOPPS (Vitrolife®, Sweden), a gamete handling medium. Secondly, samples of the plastic ART consumables that leached BPA into the G-MOPPS at a concentration above 2 ng/ml were filled for 2 min with acetonitrile (Sigma-Aldrich, USA), a typical solvent for BPA, to evaluate the release of BPA in more extreme conditions. A concentration threshold of 2 ng/ml was chosen since mean BPA concentrations in healthy women range from 0.5 to 2.5 ng/ml in serum and from 0.15 to 2.4 ng/ml in follicular fluid (Ikezuki *et al.*, 2002; Vandenberg *et al.*, 2010). Furthermore, in this case, a second batch of the consumable was tested in triplicate both in G-MOPPS and in acetonitrile to test for batch-to-batch variation. The consumables tested were filled with media (G-MOPPS or acetonitrile) at a volume close to that usually used under routine ART conditions.

The group of materials with small internal volume ($n = 10$) comprised one syringe stopper, six different pipette tips and three strippers used for oocyte denudation before ICSI or embryo handling. The stopper has no true internal volume: it was immersed in a glass tube containing 3 ml of medium which covered the entire device. The routine volumes are about 10 µl for pipette tips and 2.8 µl for strippers, considerably smaller than the minimum volume needed for our BPA measurement assay (45 µl). Therefore, we followed a two-step strategy for BPA detection. First the consumable was totally immersed in 37°C pre-warmed G-MOPPS for 2 min before 100 µl of the medium was taken for BPA detection. For pipette tips, this was done with 2.5 ml of medium in glass tubes. Regarding the strippers used for oocyte/embryo handling ($n = 3$), because of their extended length and very small diameter, they were cut into six equal pieces with a pair of metal scissors, and the pieces were plunged into a drop of 1.5 ml 37°C pre-warmed medium for 2 min in order to avoid excessive dilution. Such conditions were thought to increase the potential for BPA leaching because the entire internal and also external walls of the consumables (and the surface section in the case of strippers) may leach substances into the media, with increased leaching as duration of exposure increases. If BPA leaching above 2 ng/ml was detected on a first batch, the experiment was repeated on a second batch. This was done on three strippers per batch and two batches per brand.

For pipette tips and strippers, a second step was performed in order to mimic routine conditions of use and to test whether rinsing of these materials resulted in leaching of BPA. For each brand of pipette tips, we set the pipette at 10 µl, aspirated 10 µl of 37°C pre-warmed G-MOPPS into the tip and expelled this 10 µl into a first Eppendorf polypropylene 1.5 ml tube. Using the same tip, a second 10 µl aspirate of medium was drawn and expelled into a second Eppendorf tube, and this was repeated four more times to mimic six serial rinses of the tip. The time interval between each aspiration and expulsion was about 3 s. Then, five other tips were treated in an identical way and their aspirate added to the six Eppendorf tubes, giving a final volume of 60 µl for each of the six rinses. This was done with one batch of tips per brand. The same strategy was adopted for strippers, except that the stripper pipette was set at 2.8 µl and that we mimicked a series of 10 rinses (10 Eppendorf tubes). Therefore, 20 strippers per batch were needed to obtain a final volume of 56 µl in each of the 10 tubes. This was done on two batches of strippers per brand, yielding 60 BPA measurements using a total of 120 strippers. Throughout the process, the G-MOPPS medium was maintained in a 14 ml polypropylene tube on a warming tube device. These 14 ml tubes and the 1.5 ml Eppendorf tubes had been tested and confirmed as not leaching BPA. Finally, since two of the three strippers tested were known to be made with polycarbonate, a plastic whose synthesis requires BPA, and since the third stripper brand was made with an unspecified plastic (Table I), we tested whether these plastics contained BPA by exposing them to acetonitrile. Because of their length and their very small diameter, the strippers were cut into six equal pieces with metal scissors and plunged in a drop of 1.5 ml acetonitrile for 2 min in order to avoid excessive dilution. This was done on three strippers per batch and two batches per brand.

BPA measurement

BPA concentrations were measured according to the liquid chromatography/mass spectrometry (LC/MS) method developed for biological fluids with an Acquity UPLC system coupled with a triple quadrupole mass spectrometer (Waters Xevo, Millford, MA, USA) and adapted to the embryo culture medium (Lacroix *et al.*, 2011). Samples obtained from tested materials were half diluted with the internal standard solution (BPA-d-16 in acetonitrile at 1 µg/ml) and centrifuged for 10 min at 4°C and 20 000 g. The method was validated in terms of selectivity, linearity, repeatability, reproducibility and limit of quantification (LOQ) according to the ICH harmonized tripartite guidelines (ICH Expert Working Group, 1995). No interference was observed in blank culture medium at the retention time of BPA. The method was linear over the calibration range from 0.5 to 100 ng/ml using a linear model weighted by $1/X^2$. Within and between-day precision, as assessed with the coefficient of variation obtained from five replicates of quality control samples at three concentrations (1.5, 7.5 and 75 ng/ml), was lower than 23% and accuracy ranged from 94 to 98%. The LOQ ($n = 12$) was obtained at 0.5 ng/ml with an accuracy of 102% and the within and between-day precision expressed by the coefficient of variation was lower than 18%. In the case of detections under the LOQ, they were given a value equal to the LOQ divided by the square root of 2, as previously suggested (Hornung and Reed, 1990).

Statistical analysis

Statistical analyses were performed using Statview software v5.0 (SAS Institute, Cary, NC, USA).

Results

None of the four culture media contained BPA according to our LOQ (0.5 ng/ml). This was true in their commercial containers, in routine culture conditions (50 µl microdrops under oil at 37°C, 6% CO_2 for 24, 48 and 96 h) and whatever the type of protein supplement and concentration (data not shown).

Regarding the plastic labware with large internal volumes, these products were filled with a volume of medium very close to that used in everyday practice (Table II). We observed no leaching of BPA into the ART medium at concentration values above the 2 ng/ml threshold from any of these 23 types of plastic labwares tested in triplicate (Table II).

Regarding the plastic consumables with small internal volumes, the stopper was immersed in a glass tube containing 3 ml of medium and no BPA was detected in these conditions (Table II). For the pipette tips and strippers, whose internal volumes exceeded the minimum volume of our assay, we first tested them in conditions allowing easy sampling of this minimum volume and optimizing the possibility of BPA leaching in the medium by totally immersing them in the G-MOPPS medium for 2 min. We detected BPA leaching only from the three brands of strippers (Table III) but not from any of the six pipette tips (data not shown). The same experiment was, therefore, repeated on a second batch of strippers, and confirmed the detection of BPA above 2 ng/ml in these conditions (Table III). However, these are not appropriate conditions for mimicking the routine use of strippers and these results should not be used to reach misleading conclusions. Indeed, for the results in conditions mimicking routine ART conditions, we did not detect BPA in any of the six steps of the rinsing procedures for pipette tips (Table II). In the same way, regarding the strippers tested in conditions mimicking routine use, BPA was detected only very weakly and sporadically during the 10 rinses. In total, mean ± SD BPA concentrations (min–max values) were 0.40 ± 0.58 ng/ml (0–1.72) for Cook strippers, 0.55 ± 0.61 ng/ml (0–1.93) for Origio and 0.30 ± 0.45 ng/ml (0–1.28) for Research Instruments strippers (Table II). Five out of 60 BPA measurements fell into detectable but not quantifiable (<LOQ) ranges (2 for Cook, 1 for Origio and 2 for Research Instruments). The proportions of values above the LOQ (0.5 ng/ml) between the three brands of strippers were not statistically different (7/20 for Cook, 10/20 for Origio and 6/20 for Research Instruments strippers; $P = 0.4$, Chi-squared test). The proportions of values

Table II BPA concentrations retrieved from plastic consumables used in ART.

Product type	No.	Brand	Name	Volume used for repletion (R) or immersion (I)	BPA concentration in the culture medium (ng/ml)
Syringes for oocyte retrieval	1	BD	BD Plastipak	5 ml (R)	ND
	2	BD	BD Plastipak Luer-Lok	5 ml (R)	ND
Tubes for oocytes collection and stoppers	3	Sarstedt	Tubes for collection	6 ml (R)	ND
	4	Corning	Falcon tubes	6 ml (R)	ND
Oocyte aspiration needles and connecting tubing	5	Vitrolife	Swemed follicle aspiration set	10 ml (R)	ND
	6	Vitrolife	Extension tube	10 ml (R)	0.6 ± 0.1
	7	Origio	Single lumen needle	10 ml (R)	ND
	8	Cook Medical	Vacuum line	10 ml (R)	ND
	9	Cook Medical	Disposable extension tube	10 ml (R)	1.2 ± 0.4
Plastic dishes for embryo culture	10	Thermo Scientific	Nunc IVF Petri dishes (untreated surface)	400 µl (R)	ND
	11	Thermo Scientific	Nunc IVF Petri dishes (Nunclon treated surface)	400 µl (R)	ND
	12	Thermo Scientific	Nunc IVF 4-well dishes (untreated surface)	400 µl (R)	ND
	13	Thermo Scientific	Nunc IVF 4-well dishes (Nunclon treated surface)	400 µl (R)	ND
	14	Corning	Falcon Easy-Grip dishes	400 µl (R)	ND
	15	Corning	Falcon IVF dishes	400 µl (R)	ND
Plastic dishes for time-lapse embryo culture	16	Vitrolife/ Fertilitech	Embryoslides	400 µl (R)	ND
Catheters for embryo transfer	17	Ellios Bio Tek	Elliocath	1.3 ml (R)	<LOQ
	18	Ellios Bio Tek	Echocath	1.3 ml (R)	<LOQ
	19	Laboratoire CCD	Frydman classic catheter 4.5	1.3 ml (R)	<LOQ
	20	Laboratoire CCD	Frydman Memory standard model	1.3 ml (R)	<LOQ
	21	Cook Medical	Guardia AccessET	1.3 ml (R)	<LOQ
Syringes for embryo transfer	22	BD	BD syringe Luer-Lok	1 ml (R)	0.6 ± 0.5
	23	PentaFerte	FT 1 ml TUB SA	1 ml (R)	<LOQ
Syringe stoppers	24	B. Braun	Combi-Stoppers	3 ml (I)	ND
Pipette tips[a]	25	VWR Collection	Ultrafine tips	10 µl (R)	ND
	26	Eppendorf	Eptips biopur	10 µl (R)	ND
	27	Thermo Scientific	ART barrier tips	10 µl (R)	ND
	28	Sorenson Bioscience	Longreach pipette tips	10 µl (R)	ND
	29	Gilson	Pipetman Tips Diamond	10 µl (R)	ND
	30	Greiner Bio-One	Pipette tips	10 µl (R)	ND
Strippers for oocyte denudation[b]	31	Cook Medical	Cook Flexipet pipette	2.8 µl (R)	0.40 ± 0.58
	32	Origio	Origio stripper	2.8 µl (R)	0.55 ± 0.61
	33	Research Instruments	EZ-Tip	2.8 µl (R)	0.30 ± 0.45

Data are mean ± SD. ND, not detected; <LOQ, detected below the limit of quantification (0.5 ng/ml).
[a]Six tips per brand were used to obtain a final volume of 60 µl and tested for six serial rinses. No BPA was detected in any of the rinse steps.
[b]Twenty strippers per brand were used to obtain a final volume of 56 µl and tested for 10 serial rinses. Values are mean ± SD of the values obtained along the rinsing process since no differences were demonstrated between the rinsing steps.

above the LOQ were not significantly different (Fisher exact test) between the first five rinses and the last five rinses (3/10 versus 4/10 for Cook, 6/10 versus 4/10 for Origio and 4/10 versus 2/10 for Research Instruments). Finally, as expected from a polycarbonate material, plunging the whole Cook and Origio strippers into a BPA solvent such as acetonitrile revealed that their plastic contained BPA (Table III). The Research Instruments strippers, whose plastic composition was not known, also leached BPA when exposed to acetonitrile (Table III).

Discussion

This is the largest study yet performed on BPA detection in the media or in the leachate of materials used in ART. Four embryo culture media, 3 types of protein supplements and 33 plastic labware products were tested for containing or leaching BPA. As far as we know, there are no available data regarding the concentration of BPA in human oviductal and/or uterine secretions. Therefore, we cannot compare our results with a physiological situation.

Table III BPA concentrations retrieved after a 2-min exposure to 37°C pre-warmed G-MOPPS medium or to acetonitrile from two batches of stripper (three strippers tested per batch) from three different brands.

Product type	No.	Brand	Name	BPA concentration in culture medium[a] (ng/ml)		BPA concentration in acetonitrile[a] (ng/ml)	
				Batch 1	Batch 2	Batch 1	Batch 2
Strippers for oocyte denudation	31	Cook Medical	Cook Flexipet pipette	3.4 ± 0.6	3.3 ± 0.1	20.5 ± 5.2	79.9 ± 22.3
	32	Origio	Origio stripper	4.1 ± 2.0	10.2 ± 6.0	8.1 ± 3.5	62.6 ± 20.0
	33	Research Instruments	EZ-Tip	2.4 ± 0.9	4.4 ± 3.8	6.4 ± 1.5	33.1 ± 9.3

Data are mean ± SD.
[a]In a volume of 1.5 ml after the strippers were cut into six equal parts for total immersion of the device.

A reassuring finding is that no BPA could be detected in the different culture media tested, either in their commercial containers or in conditions of routine use. This confirms a previous result on one commercial embryo culture medium (G-1^{TM} v5, Vitrolife, Mahalingaiah *et al.*, 2012). Furthermore, we could not detect BPA after protein supplementation at 5 or 10%. This is a particularly important point for purified HSA supplementation, since it is known that BPA can bind to HSA (Xie *et al.*, 2010) or bovine serum albumin (Zhang *et al.*, 2010) and it is highly suspected that the addition of insufficiently purified HSA in human embryo culture media is the major source for introducing a large variety of undesired molecules at low levels, such as proteins (Dyrlund *et al.*, 2014) and phthalates (Takatori *et al.*, 2012).

Furthermore, we demonstrated that among the various plastic consumables used for ART, none released BPA into the culture medium at concentrations exceeding those usually reported in normal environmental conditions in human serum (0.5–2.5 ng/ml) or follicular fluid (0.15–2.4 ng/ml) (Ikezuki *et al.*, 2002; Vandenberg *et al.*, 2010). Unfortunately we had no estimates of serum or follicular fluid BPA levels in women obtained using the same LC/MS method and therefore can only compare our data to values reported in the literature. In the mouse, experiments *in vitro* have shown that a range of concentrations of BPA can influence embryo development (as an inverse U-shaped curve). BPA has no effect on mouse preimplantation development at a very weak concentration (0.1 nM, or 0.023 ng/ml, Takai *et al.*, 2000), but it can increase the proportion of 2-cell embryos reaching the 8-cell stage after 24 h of culture and the blastocyst stage after 48 h of culture at a low but environmentally relevant concentration of BPA (1–3 nM, or 0.23–0.69 ng/ml, Takai *et al.*, 2000, 2001), while a 10 nM (2.3 ng/ml) concentration of BPA in the culture medium had no effect on embryo developmental kinetics in the mouse (Takai *et al.*, 2000). Lastly, a high BPA concentration strongly decreases the blastocyst rate (100 μM, or 22.8 μg/ml, Takai *et al.*, 2000, 2001; Lee *et al.*, 2012). The very few plastic consumables for which BPA was detected in the culture medium leached BPA into the surrounding medium in concentrations of a few tenths of ng/ml (from 0.3 to 1.2 ng/ml, Table II). Importantly, *in vitro* exposure of embryos to any substance at a concentration encountered *in vivo* does not mean that this exposure mimics the physiological state, unless we deny that oviductal and endometrial environments play any role. The latter are considered not only to supply the best nutritive and trophic factors to embryos but also to protect them from injury, for example through their detoxifying properties. The deleterious effect on mouse blastocyst rate of high BPA concentrations (100 μM) added to *in vitro* culture medium was significantly reduced when the culture was performed on human endometrial epithelial cells (Lee et al., 2012). The low levels of BPA we have detected in this study are reassuring and are clearly lower than those described *in vivo*. It should also be borne in mind that after embryos are stripped, they are very rapidly flushed out in fresh medium. Nevertheless, our results raise questions since during ART procedures human embryos are not protected by the uterine environment, and there are no data, either *in vitro* or *in vivo*, regarding the toxicological thresholds of BPA for human preimplantation embryos. In acetonitrile solvent, the three strippers used for oocyte denudation and/or gamete or embryo manipulation were shown to contain BPA, to leach between 2 and 10 ng/ml BPA when cut and totally immersed in the culture medium for 2 min (i.e. in non-realistic conditions that do not mimic routine work) and finally, to sporadically leach BPA at low levels (a few tenths of ng/ml) in routine conditions. A very important point is that two out of three strippers are made of polycarbonate plastic, according to the manufacturers' information (Table I). Polycarbonate synthesis is well known to require BPA since it is essentially a polymer of BPA and is widely used for manufacturing ART strippers because it is reputed to be unbreakable. This could explain why we detected very low BPA levels even after several rounds of rinsing: the entire thickness of the stripper is a BPA polymer that is capable in theory of leaching BPA at any time. Concerning the third stripper brand for which we also detected BPA (EZ-Tip, Research Instruments, UK), we could not find any information about the plastic used for its manufacture. However, since exposure of this stripper to acetonitrile revealed BPA at concentrations close to those found for the other two brands, we can hypothesize that it is also made with polycarbonate. In our study, the only other consumable made with polycarbonate was a 1 ml syringe used for embryo transfer (No. 22, Tables I and II). In this syringe, the body is made of polycarbonate and the piston is made of polypropylene, while epoxy resin (a potential source of BPA) is used to glue the seal to the piston. We detected quantifiable but low BPA leaching (0.6 ng/ml) from these syringes. To confirm that BPA was present in the plastics, we filled the syringes with acetonitrile (1 ml). As expected, higher BPA concentrations were detected in acetonitrile (17.5 ng/ml). Interestingly, other syringes from the same brand but whose body and piston are made with polypropylene did not leach detectable BPA (No. 1 and 2, Tables I and II), indicating that the plastic used for the syringe body was the source of BPA, rather than the epoxy resin glue.

Unlike embryo culture dishes, previously excluded as a BPA source by other authors (Mahalingaiah *et al.*, 2012), most plastic ART labware products are not in prolonged contact with embryos. Therefore, it may be questioned whether brief contact (about 1 min) is sufficient to have

consequences on oocyte/embryo development or physiology, even in the event of high BPA leaching. Physiological signals, such as glucose, are known to have a rapid action on embryo physiology, as a 1-min exposure to glucose during preimplantation development has been shown to overcome morula block in the mouse embryo (Chatot *et al.*, 1994). For BPA, although ER expression does occur in early embryos (Hiroi *et al.*, 1999) and could play a role in zygotic genome activation (Zhang *et al.*, 2015), such rapid action would preclude the classic mechanism of BPA genomic action via ER binding to the cytoplasm or nucleus to initiate the transcription and translation of ER-responsive genes. Instead, it would imply a non-genomic action via ER membrane binding. It has in fact been shown that dormant mouse blastocysts can be reactivated after brief exposure (20 min) to estradiol via such a non-genomic mechanism (Yu *et al.*, 2009). Non-genomic action of BPA has been reported in many cell types (Nadal *et al.*, 2000; Thomas and Dong, 2006; Watson *et al.*, 2007; Bouskine *et al.*, 2009) but this knowledge is lacking for the mammalian preimplantation embryo. Therefore, we can only speculate that human embryos would be affected by brief contact with high levels of BPA (not demonstrated in this work) during a manipulation. We emphasize, however, that it has been elegantly shown that the embryo transfer procedure *per se* can induce alterations of the epigenome in the post-implantation embryo and placenta, thus leading to placental dysfunctions (Rivera *et al.*, 2008).

One intriguing point of our results is that the levels of detection of BPA after exposure to acetonitrile differed between the two batches of strippers for the three brands of strippers. We have no clear explanation for this difference. Although the two batches were tested on different runs of the assay, the between-run precision was acceptable (<23%) and does not explain the difference. A possible explanation could be the way the strippers were cut before exposure: the length of the six pieces could vary between experiments, and the scissors used were not identical and could have crushed the plastic in different ways. Another explanation could relate to the age of the batch (date of manufacture) or to storage conditions. However, since most of the strippers were contributed by other French ART laboratories, we have no access to such information and are unable to verify this possibility. Therefore, the differences between batches observed here should be taken with caution until further analyses are performed. Finally, they could also reflect differences in the manufacturing of the two batches, regarding either the plastic used or contamination by a source of BPA during manufacturing, or a difference of position of the tested specimens within each batch leading to varying dilution of a BPA source during the manufacturing process of each batch. It should be stressed that it is not surprising to observe differences between batches of ART plastic consumables. Toxicological tests (such as mouse embryo assays or sperm survival tests) for screening such consumables have in fact been introduced as a routine procedure in some ART centers as these tests can result in exclusion of about 10% of tested batches (Punt-van der Zalm *et al.*, 2009; Nijs *et al.*, 2009). Furthermore, a 10-fold difference in the aluminum concentration in two batches of a protein supplement for human embryo culture has recently been reported (Morbeck *et al.*, 2014).

In conclusion, it appears that although BPA is a constituent of a few plastic consumables used in ART procedures, none of them leach BPA into the surrounding medium at concentrations above those measured previously in serum or follicular fluid in women. Concerning the epigenetic effects of BPA in animals and *in vitro* studies, the European Food Safety Authority (EFSA) expert panel recently concluded that the current data 'should not be neglected but considered as an indication that BPA in principle has the potential to alter the epigenome' (EFSA, 2015). Since maternal exposure to environmentally relevant doses of BPA has been shown to affect DNA methylation level in the mouse early embryo and placenta only if exposure takes place during periconception, i.e. during the stage of extensive epigenetic reprogramming of the genome (Susiarjo *et al.*, 2013), we may question the use of polycarbonate plastic to manufacture any ART plastic consumable. Strikingly, we note that two major suppliers of ART consumables (Origio, Vitrolife) propose borosilicate glass strippers. This alternative may perhaps appear as an excessive application of the precautionary principle, but it should be considered especially while time-lapse embryo culture is developing and requires the use of strippers for embryo handling. Finally, further studies are needed to decipher the presence and potential effects of plastic toxicants on human embryos during ART procedures.

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Authors' roles

N.G., M.Z.L. and S.C. participated in the execution and analysis of the study, N.G. and M.Z.L. also took part in critical discussion of the draft manuscript. N.P.-H. and V.G. participated in analysis and critical discussion of the draft. J.P. participated in the design of the study and its writing. R.D.L. designed the study, participated in its execution and analysis, and wrote the manuscript.

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Conflict of interest

None declared.

References

Bouskine A, Nebout M, Brucker-Davis F, Benahmed M, Fenichel P. Low doses of bisphenol A promote human seminoma cell proliferation by activating PKA and PKG via a membrane G-protein-coupled estrogen receptor. *Environ Health Perspect* 2009;**117**:1053–1058.

Buck Louis GM, Sundaram R, Sweeney AM, Schisterman EF, Maisog J, Kannan K. Urinary bisphenol A, phthalates, and couple fecundity: the Longitudinal Investigation of Fertility and the Environment (LIFE) Study. *Fertil Steril* 2014;**101**:1359–1366.

Calafat AM, Ye X, Wong LY, Reidy JA, Needham LL. Exposure of the U.S. population to bisphenol A and 4-tertiary-octylphenol: 2003-2004. *Environ Health Perspect* 2008;**116**:39–44.

Calafat AM, Weuve J, Ye X, Jia LT, Hu H, Ringer S, Huttner K, Hauser R. Exposure to bisphenol A and other phenols in neonatal intensive care unit premature infants. *Environ Health Perspect* 2009;**117**:639–644.

Caserta D, Di Segni N, Mallozzi M, Giovanale V, Mantovani A, Marci R, Moscarini M. Bisphenol A and the female reproductive tract: an overview of recent laboratory evidence and epidemiological studies. *Reprod Biol Endocrinol* 2014;**12**:37.

Chatot CL, Lewis-Williams J, Torres I, Ziomek CA. One-minute exposure of 4-cell mouse embryos to glucose overcomes morula block in CZB medium. *Mol Reprod Dev* 1994;**37**:407–412.

Duty SM, Mendonca K, Hauser R, Calafat AM, Ye X, Meeker JD, Ackerman R, Cullinane J, Faller J, Ringer S. Potential sources of bisphenol A in the neonatal intensive care unit. *Pediatrics* 2013;**131**:483–489.

Dyrlund TF, Kirkegaard K, Poulsen ET, Sanggaard KW, Hindkjaer JJ, Kjems J, Enghild JJ, Ingerslev HJ. Unconditioned commercial embryo culture media contain a large variety of non-declared proteins: a comprehensive proteomics analysis. *Hum Reprod* 2014;**29**:2421–2430.

Ehrlich S, Williams PL, Missmer SA, Flaws JA, Ye X, Calafat AM, Petrozza JC, Wright D, Hauser R. Urinary bisphenol A concentrations and early reproductive health outcomes among women undergoing IVF. *Hum Reprod* 2012a;**27**:3583–3592.

Ehrlich S, Williams PL, Missmer SA, Flaws JA, Berry KF, Calafat AM, Ye X, Petrozza JC, Wright D, Hauser R. Urinary bisphenol A concentrations and implantation failure among women undergoing in vitro fertilization. *Environ Health Perspect* 2012b;**120**:978–983.

European Food Safety Authority (EFSA). Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs: Part II – Toxicological assessment and risk characterisation. *EFSA Journal* 2015;**13**:3978.

Geens T, Aerts D, Berthot C, Bourguignon JP, Goeyens L, Lecomte P, Maghuin-Rogister G, Pironnet AM, Pussemier L, Scippo ML *et al.* A review of dietary and non-dietary exposure to bisphenol-A. *Food Chem Toxicol* 2012;**50**:3725–3740.

Hiroi H, Momoeda M, Inoue S, Tsuchiya F, Matsumi H, Tsutsumi O, Muramatsu M, Taketani Y. Stage-specific expression of estrogen receptor subtypes and estrogen responsive finger protein in preimplantational mouse embryos. *Endocr J* 1999;**46**:153–158.

Hong J, Chen F, Wang X, Bai Y, Zhou R, Li Y, Chen L. Exposure of preimplantation embryos to low-dose bisphenol A impairs testes development and suppresses histone acetylation of StAR promoter to reduce production of testosterone in mice. *Mol Cell Endocrinol* 2016. doi: 10.1016/j.mce.2016.03.009 (epub ahead of print).

Hornung RW, Reed LD. Estimation of average concentration in the presence of nondetectable values. *Appl Occup Environ Hyg* 1990;**5**:46–51.

Howdeshell KL, Hotchkiss AK, Thayer KA, Vandenbergh JG, vom Saal FS. Exposure to bisphenol A advances puberty. *Nature* 1999;**401**: 763–764.

Huygh J, Clotman K, Malarvannan G, Covaci A, Schepens T, Verbrugghe W, Dirinck E, Van Gaal L, Jorens PG. Considerable exposure to the endocrine disrupting chemicals phthalates and bisphenol-A in intensive care unit (ICU) patients. *Environ Int* 2015;**81**:64–72.

ICH Expert Working Group. Validation of analytical procedures: test and methodology Q2(R1). *International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use*. 1995.

Ikezuki Y, Tsutsumi O, Takai Y, Kamei Y, Taketani Y. Determination of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure. *Hum Reprod* 2002;**17**:2839–2841.

Lacroix MZ, Puel S, Collet SH, Corbel T, Picard-Hagen N, Toutain PL, Viguie C, Gayrard V. Simultaneous quantification of bisphenol A and its glucuronide metabolite (BPA-G) in plasma and urine: applicability to toxicokinetic investigations. *Talanta* 2011;**85**:2053–2059.

Lee MS, Lee YS, Lee HH, Song HY. Human endometrial cell coculture reduces the endocrine disruptor toxicity on mouse embryo development. *J Occup Med Toxicol* 2012;**7**:7.

Machtinger R, Combelles CM, Missmer SA, Correia KF, Williams P, Hauser R, Racowsky C. Bisphenol-A and human oocyte maturation in vitro. *Hum Reprod* 2013;**28**:2735–2745.

Mahalingaiah S, Hauser R, Patterson DG Jr., Woudneh M, Racowsky C. Bisphenol A is not detectable in media or selected contact materials used in IVF. *Reprod Biomed Online* 2012;**25**:608–611.

Mok-Lin E, Ehrlich S, Williams PL, Petrozza J, Wright DL, Calafat AM, Ye X, Hauser R. Urinary bisphenol A concentrations and ovarian response among women undergoing IVF. *Int J Androl* 2010;**33**:385–393.

Morbeck DE, Paczkowski M, Fredrickson JR, Krisher RL, Hoff HS, Baumann NA, Moyer T, Matern D. Composition of protein supplements used for human embryo culture. *J Assist Reprod Genet* 2014; **31**:1703–1711.

Nadal A, Ropero AB, Laribi O, Maillet M, Fuentes E, Soria B. Nongenomic actions of estrogens and xenoestrogens by binding at a plasma membrane receptor unrelated to estrogen receptor alpha and estrogen receptor beta. *Proc Natl Acad Sci USA* 2000;**97**:11603–11608.

Nijs M, Franssen K, Cox A, Wissmann D, Ruis H, Ombelet W. Reprotoxicity of intrauterine insemination and in vitro fertilization-embryo transfer disposables and products: a 4-year survey. *Fertil Steril* 2009;**92**:527–535.

Peretz J, Vrooman L, Ricke WA, Hunt PA, Ehrlich S, Hauser R, Padmanabhan V, Taylor HS, Swan SH, VandeVoort CA *et al.* Bisphenol a and reproductive health: update of experimental and human evidence, 2007–2013. *Environ Health Perspect* 2014;**122**:775–786.

Punt-van der Zalm JP, Hendriks JC, Westphal JR, Kremer JA, Teerenstra S, Wetzels AM. Toxicity testing of human assisted reproduction devices using the mouse embryo assay. *Reprod Biomed Online* 2009;**18**: 529–535.

Richter CA, Birnbaum LS, Farabollini F, Newbold RR, Rubin BS, Talsness CE, Vandenbergh JG, Walser-Kuntz DR, vom Saal FS. In vivo effects of bisphenol A in laboratory rodent studies. *Reprod Toxicol* 2007; **24**:199–224.

Rivera RM, Stein P, Weaver JR, Mager J, Schultz RM, Bartolomei MS. Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development. *Hum Mol Genet* 2008;**17**:1–14.

Souter I, Smith KW, Dimitriadis I, Ehrlich S, Williams PL, Calafat AM, Hauser R. The association of bisphenol-A urinary concentrations with antral follicle counts and other measures of ovarian reserve in women undergoing infertility treatments. *Reprod Toxicol* 2013;**42**:224–231.

Susiarjo M, Sasson I, Mesaros C, Bartolomei MS. Bisphenol A exposure disrupts genomic imprinting in the mouse. *PLoS Genet* 2013;**9**:e1003401.

Takai Y, Tsutsumi O, Ikezuki Y, Hiroi H, Osuga Y, Momoeda M, Yano T, Taketani Y. Estrogen receptor-mediated effects of a xenoestrogen, bisphenol A, on preimplantation mouse embryos. *Biochem Biophys Res Commun* 2000;**270**:918–921.

Takai Y, Tsutsumi O, Ikezuki Y, Kamei Y, Osuga Y, Yano T, Taketan Y. Preimplantation exposure to bisphenol A advances postnatal development. *Reprod Toxicol* 2001;**15**:71–74.

Takatori S, Akutsu K, Kondo F, Ishii R, Nakazawa H, Makino T. Di(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate in media for in vitro fertilization. *Chemosphere* 2012;**86**:454–459.

Thomas P, Dong J. Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption. *J Steroid Biochem Mol Biol* 2006; **102**:175–179.

Vandenberg LN, Chahoud I, Heindel JJ, Padmanabhan V, Paumgartten FJ, Schoenfelder G. Urinary, circulating, and tissue biomonitoring studies

indicate widespread exposure to bisphenol A. *Environ Health Perspect* 2010;**118**:1055–1070.

Watson CS, Bulayeva NN, Wozniak AL, Alyea RA. Xenoestrogens are potent activators of nongenomic estrogenic responses. *Steroids* 2007; **72**:124–134.

WHO. Toxicological and Health Aspects of Bisphenol A. Report of Joint FAO/WHO Expert Meeting. Ottawa, Canada: World Health Organization, 2011, 1–60.

Xie X, Wang X, Xu X, Sun H, Chen X. Investigation of the interaction between endocrine disruptor bisphenol A and human serum albumin. *Chemosphere* 2010;**80**:1075–1080.

Yu LL, Zhang JH, He YP, Huang P, Yue LM. Fast action of estrogen on intracellular calcium in dormant mouse blastocyst and its possible mechanism. *Fertil Steril* 2009;**91**:611–615.

Zhang YL, Zhang X, Fei XC, Wang SL, Gao HW. Binding of bisphenol A and acrylamide to BSA and DNA: insights into the comparative interactions of harmful chemicals with functional biomacromolecules. *J Hazard Mater* 2010;**182**:877–885.

Zhang Y, Jiang Y, Lian X, Xu S, Wei J, Chu C, Wang S. Effects of ERalpha-specific antagonist on mouse preimplantation embryo development and zygotic genome activation. *J Steroid Biochem Mol Biol* 2015;**145**:13–20.