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Endocrine-disrupting chemicals in human follicular fluid impair *in vitro* oocyte developmental competence

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BACKGROUND: Increased global industrial activity has exposed humans to a wide variety of chemical substances some of which, called 'endocrine-disrupting chemicals' (EDCs) or 'endocrine disruptors', can disrupt the endocrine system in the body. The ovarian follicle is a very fragile micro-environment where interactions between hormones, growth factors, the oocyte and its surrounding somatic cells are essential to generate a fully competent oocyte. *In vitro* experiments suggest that EDCs can disturb this finely tuned balance, but very scarse *in vivo* data are available to confirm this assumption. Therefore, we have investigated if the presence of EDCs in human follicular fluid is a risk factor for the developmental competence of an *in vivo* exposed oocyte. Furthermore, because of the limited access to human follicular fluid, we verified if follicular fluid contamination can be predicted based on EDC levels in serum.

METHODS: Follicular fluid ($n = 40$) and serum ($n = 20$) samples from women undergoing assisted reproductive technology (ART) were analyzed by means of gas chromatography combined with mass spectrometry to examine the presence of different EDCs, such as polychlorinated biphenyls, polybrominated diphenyl ethers and organochlorine pesticides. Statistical models were used to investigate the relation between the characteristics and ART results of the patients and the contamination status of their follicular fluid and to assess the capacity of serum samples to predict follicular fluid contamination.

RESULTS: Chlorinated biphenyl 153 (72 ± 44 and 201 ± 106 pg/ml) and *p,p'*-DDE (392 ± 348 and 622 ± 406 pg/ml) were the compounds found in the highest concentrations in follicular fluid and serum samples, respectively. A new variable principal component 1, representing the overall contamination status of the follicular fluid samples, is strongly associated with fertilization rate ($P < 0.00001$) and the proportion of high-quality embryos relative to the amount of retrieved oocytes ($P < 0.05$), even when the analysis is adjusted for age, estradiol concentration, BMI, fertilization procedure and male subfertility as explanatory variables. The strong correlations between the EDC concentrations in serum and follicular fluid ($r \geq 0.93$) allowed us to build regression models, which accurately predict EDC concentrations in the follicular fluid based on serum samples.

CONCLUSIONS: An overall higher EDC contamination in the follicular micro-environment was associated with a decreased fertilization rate and consequently with a lower chance of an oocyte to develop into a high-quality embryo. In addition, EDC concentrations in serum were reliable predictors of the contamination status of the follicular micro-environment.

Key words: endocrine disruptors / oocyte quality / follicular fluid / female subfertility / serum

Introduction

Since the industrial revolution, the release of chemicals into the environment has significantly increased. However, it was only after the publication of Rachel Carson's book 'Silent Spring' in 1962 that attention was drawn to the possible toxic effects of these chemicals on our ecosystem (Carson, 1962). This growing awareness resulted in a ban of multiple pesticides (e.g. dichlorodiphenyltrichloroethane, DDT) and other chemicals (e.g. polychlorinated biphenyls, PCBs) in many developed countries during the seventies (Colborn *et al.*, 1993). Nevertheless, various banned substances are still detected in our environment because of their long half-lives (Covaci *et al.*, 2002; Jaspers *et al.*, 2006; Josefsson *et al.*, 2011). Additional research revealed that some of these chemicals were able to interfere with the synthesis, function, storage and/or metabolism of hormones (Sweeney, 2002). As a result of this ability to interact with the endocrine system, these substances were denominated as 'endocrine-disrupting chemicals' (EDCs) or 'endocrine disruptors' (Colborn *et al.*, 1993). Furthermore, due to their lipophilic and persistent characteristics, EDCs undergo the process of bio-accumulation, with the highest concentrations found in species at the top of the food chain (e.g. human; Jaspers *et al.*, 2006; Wang and Needham, 2007; Covaci *et al.*, 2008).

Wildlife observations in highly contaminated geographical areas revealed that EDCs can cause reproductive abnormalities (Vos *et al.*, 2000; Bernanke and Kohler, 2009; Hamlin and Guillette, 2010). Alligator ovaries for example exhibited increased numbers of multi-oocyte follicles and polynuclear oocytes after a large pesticide spill with primarily dicofol and DDT derivatives (Milnes and Guillette, 2008). Subsequently, laboratory animal experiments confirmed the ability of EDCs to induce reproductive disorders (Diamanti-Kandarakis *et al.*, 2009). Although it remains unclear whether EDCs can execute comparable effects in humans (Ross, 2004; Vandenberg *et al.*, 2009), they have already been suggested as a potential key player in human subfertility (Toft *et al.*, 2004; Diamanti-Kandarakis *et al.*, 2009). Within the reproductive system, the ovarian follicle can be considered as a very fragile micro-environment where interactions between hormones, growth factors, the oocyte and its surrounding somatic cells are essential to generate a fully competent oocyte. Disruption of this finely tuned (endocrine/paracrine) balance can lead to anovulation (Mumford *et al.*, 2011), cystic deformation (Baptiste *et al.*, 2010) or a diminished oocyte quality which jeopardizes further embryo development (Leroy *et al.*, 2005). Although *in vitro* experiments suggest a role for EDCs in disturbing the tightly regulated endocrine and paracrine signaling in the different cells of the ovarian follicle (Brevini *et al.*, 2004; Pocar *et al.*, 2006; Kwintkiewicz *et al.*, 2010), these exposure experiments can only be related to the *in vivo* situation if environmental relevant EDC concentrations are considered. Moreover, almost no *in vivo* data are currently available about the association of a women's IVF record and the presence of EDCs in her follicular fluid, i.e. the *in vivo* micro-environment in which the female gamete grows and matures. Knowledge about the contamination status of human follicular fluid is thus indispensable, which implies the continuous need for monitoring EDC concentrations in the follicular fluid (Trapp *et al.*, 1984; Jarrell *et al.*, 1993; Pauwels *et al.*, 1999; Younglai *et al.*, 2002; Weiss *et al.*, 2006; Meeker *et al.*, 2009; Jirsova *et al.*, 2010). In addition, due to the complicated sampling procedure and to ethical requirements, follicular fluid can only be obtained on a regular basis in assisted reproductive technology (ART) settings. The possibility to predict EDC concentrations in the follicular fluid based on serum measurements would therefore be of a great value.

In the present study, we investigate if the presence of EDCs in human follicular fluid is a risk factor for a reduced developmental competence of the *in vivo* exposed oocyte. Consequently, the aims of this study were (i) to assess the presence of the most common EDCs in the follicular fluid from ART patients; (ii) to examine a potential link between these concentrations and the patient's characteristics and ART outcome and (iii) to examine the option of using serum samples to predict EDC concentrations in the follicular fluid.

Materials and Methods

Sample collection

During two identical sampling periods, human follicular fluid samples ($n = 20$, March 2008) and paired serum and follicular fluid samples ($n = 20$, March–May 2009) were collected in the fertility unit of ZNA Middelheim Hospital, Antwerp, Belgium, following the approval of the ethical committees from the University of Antwerp and ZNA Middelheim Hospital. All patients signed informed consent papers. During routine ART procedures, patients were stimulated daily with HMG (Menopur®, Ferring Pharmaceuticals, Copenhagen, Denmark) or FSH (Gonal-F®, Merck Serono, Geneva, Switzerland or Puregon®, MSD, Oss, The Netherlands) at a dose of 150–225 IU/day for ~14 days. Patients received a long GnRH-agonist protocol whereby busereline (0.1 mg/dose, Suprefact®, Sanofi-Aventis, Frankfurt, Germany) was administered six times a day intranasally from 3 weeks before the beginning of the stimulation protocol until the moment of ovum pick-up (OPU). When at least three follicles were >17 mm, 10 000 IU hCG (Pregnyl®, MSD, Oss, The Netherlands) was administered. Oocytes were retrieved 37 h later by means of ultrasound-guided follicular aspiration. To prevent contamination with blood or flushing medium, only follicular fluid from the first punctured follicle was collected. Venous blood samples were taken immediately following the ART procedure. Follicular fluid and coagulated blood samples were centrifuged (1500 *g*, 20 min and 1400 *g*, 30 min, respectively) and the supernatant was stored in polypropylene tubes at −20°C until analysis.

Patient information and ART characteristics

In our study group, age, height, weight, occupation and residence were collected from the patients and consequently body mass index (BMI, kg/m^2) was calculated. Furthermore, the following cycle parameters were collected: serum estradiol concentration on the day of hCG administration estradiol concentration [E2] (i.e. 37 h before OPU), number of retrieved oocytes, number of zygotes with two pronuclei (2PN-zygotes, 18 h after insemination/injection), number of high-quality embryos (Table I), fertilization procedure (IVF or ICSI), initial subfertility problem, ART- and pregnancy outcome. Embryos were characterized as a high- or top-quality embryo in the absence of multinucleated blastomeres, 4–5 blastomeres on Day 2, ≥7 blastomeres on Day 3 and ≤20% anucleated fragments (Van Royen *et al.*, 1999). With these data, the fertilization rate (number of 2PN-zygotes/number of retrieved oocytes) and the proportion of top-quality embryos of a patient relative to the amount of retrieved oocytes as well as to the amount of 2PN-zygotes (number of high-quality embryos/number of retrieved oocytes and number of high-quality embryos/number of 2PN-zygotes, respectively) could be calculated. An ART procedure was defined as successful if a gestational sac with positive heart beat was detected around 7 weeks amenorrhea. The birth of one or more healthy babies was considered as a successful pregnancy.

Table I Characteristics and IVF record of the 40 participating patients

	Mean	SD	Median	Min–Max
Characteristics				
Age (years)	34.5	4.4	34.1	25.2–42.8
Height (cm)	167	6.6	168	155–182
Body weight (kg)	64.8	15.4	62.0	45–117
BMI (kg/m^2)	23.1	4.8	22.1	16.9–38.2
[E2][a] (pg/ml)	1847	1129	1677	253–4572
No. of retrieved oocytes	9	5	8	2–28
No. of 2PN-zygotes[b]	6	4	5	0–24
Fertilization rate[c]	0.72	0.26	0.76	0–1
No. of top quality embryos[d]	2	3	1	0–11
Proportion of top-quality embryos[e]	0.22	0.21	0.17	0–0.71
Outcomes	%			
Clinical pregnancy[f]	30			
Live birth[g]	25			

BMI, body mass index.
[a][E2], serum estradiol concentration on the day of hCG administration (i.e. 37 h before OPU).
[b]Number of zygotes with two pronuclei.
[c]Number of zygotes with two pronuclei/retrieved oocytes.
[d]Van Royen *et al.*, 1999.
[e]Number of top-quality embryos/retrieved oocytes.
[f]Detection of gestational sac with positive heart beat (after 7 weeks amenorrhea).
[g]Birth of one or more healthy babies.

Chemical analysis of body fluids

In all samples, 26 PCB congeners, 7 polybrominated diphenyl ether (PBDE) congeners, and the pesticides hexachlorobenzene (HCB), chlordanes (*cis*-chlordane, *trans*-chlordane, *trans*-nonachlor and oxychlordane), hexachlorocyclohexanes (α-, β-, γ-HCHs), *p*,*p*′-DDT and its metabolites (*pp*′-DDD and *p*,*p*′-DDE) were analyzed. Serum and follicular fluid samples were treated as previously described following a slightly adjusted method (Covaci and Schepens, 2001). All standards were obtained from Dr. Ehrenstorfer Laboratories (Augsburg, Germany). All solvents were of pesticide-grade purity and were, together with concentrated sulfuric acid (analytical grade) and silica gel 60 (63–230 mesh), available from Merck (Darmstadt, Germany). In short, samples (3–4 ml) were spiked with internal standards, mixed with deionized water and formic acid (used for protein denaturation) and, after ultrasonication, loaded on C18 Empore™ solid-phase disk extraction cartridges (3M Company, St. Paul, MN, USA). After elution, the extracts were cleaned on acidified silica and analytes were further eluted with hexane and dichloromethane. The cleaned eluate was then concentrated to near dryness, re-dissolved in *iso*-octane and analyzed by gas chromatography coupled to mass spectrometry (GC/MS). The GC/MS was operated in electron-capture negative ionization (ECNI) mode, using a DB-5 capillary column (30 m × 0.25 mm × 0.25 μm).

Multi-level calibration curves ($r^2 > 0.999$) in the linear response interval of the detector were created for the quantification. The quality control was performed by regular analyses of procedural blanks, by random injection of standards, spiked samples and solvent blanks. Procedural blanks were consistent (<15%) and therefore the mean procedural blank value was used for subtraction. After blank subtraction, the method limit of quantification (LOQ) was set at 3 × standard deviation (SD) of the blank (which ensures >99% certainty that the reported value is originating from the sample) taking into consideration the volume of the analyzed sample. The quality control scheme was also assessed through regular participation to inter-laboratory comparison exercises organized by the Arctic Monitoring and Assessment Program (AMAP; persistent organic pollutants in human serum; AMAP, 2010). Obtained values were deviating with <20% from the consensus values.

The total amount of lipids (TL) in the serum samples was calculated using the following formula: TL (g/l) = 2.27 × total cholesterol + triglycerides + 0.62 (Phillips *et al.*, 1989).

Data treatment

Statistical analysis was performed using SPSS 15.0 for Windows (Chicago, IL, USA) and R version 2.13.0 (R Development Core Team, 2008). Values of $P < 0.05$ were considered as statistically significant. Samples with levels below the LOQ were assigned a value of $f \times$ LOQ with f being the detection frequency or the ratio between the number of samples detected above the method LOQ and the total number of analyzed samples (Voorspoels *et al.*, 2002). By doing so, data below LOQ can still be used in the statistical data treatment. However, compounds with <50% of the measurements above their LOQs were excluded from statistical analysis.

Data are presented as mean with SD. Serum samples are expressed both per volume (/ml) and lipid normalized [per g lipid weight (g lw)]. Normality was checked using the Shapiro–Wilk test. Because of the relative low number of samples in the study and the detection of outliers, the Wilcoxon matched-pairs signed-rank test was used to examine the difference in compound concentrations between the serum and follicular fluid. Correlations between follicular fluid and serum concentrations and patient characteristics were investigated using the Spearman rank test. Regression analysis was carried out on both untransformed (linear) as well as log-transformed concentrations to examine how accurately EDC concentrations in the follicular fluid can be estimated based on serum concentrations. Principal components analysis (PCA), a data reduction technique that re-organizes the nine original variables into nine new variables, called principal components (PCs), was performed on the correlation matrix. Subsequently, scores were calculated for the first PC. These PC scores were then entered in logistic regression analysis to test their association with the fertilization rate, proportion of top-quality embryos relative to both the number of retrieved oocytes and the number of 2PN-zygotes, ART- and pregnancy outcome. For all these outcomes, significance of the PC scores was tested by a likelihood ratio test, comparing a model with the PC scores, age, [E2], BMI, fertilization procedure and the fact if male subfertility was the initial reason to start with ART, to a null model only containing these latter five variables. This procedure was executed for all the variables. When the outcome was a proportion (fertilization rate and proportion of high-quality embryos relative to both the number of retrieved oocytes and 2PN-zygotes), logistic regression was performed as described in Venables and Ripley (Modern Applied Statistics in S, fourth edition, pp. 191–192, Springer). Basically, the outcome variable is ordered into a two-column matrix, with the first column giving the number of successes and the second column the number of failures for each individual. For instance, to perform the analysis on the proportion of high-quality embryos relative to the total number of ova, the first column would contain the number of high-quality embryos (success), with the second column containing the number of ova that failed to become a high-quality embryo.

Results

Patients ($n = 40$) were on average 34.5 ± 4.4 years old, had an average BMI of 23.1 ± 4.8 kg/m^2 (Table I) and they all lived in the urbanized area. None of the patients from which information about their occupation was available ($n = 14$) worked in a high-risk exposure environment. IVF was used in the majority of patients (58%), the others were treated with ICSI. Of all ART procedures in this study, 30% achieved an ongoing pregnancy and 25% achieved a live birth. Several compounds (CBs 118, 138, 153, 170, 180, HCB, β-HCH and *p*,*p*′-DDE) were present in nearly every serum and follicular fluid sample, whereas other chemicals had a detection frequency of <50% and were excluded from statistical analysis (Table II). Other compounds could not be detected above their respective LOQs in both the serum and follicular fluid samples (CBs 28/31, 52, 74, 95, 99, 101, 110, 128, 132, 149, 156, 167, 194, 196/203, 199; BDEs 28, 100, 153, 154, 183; *cis*- and *trans*-chlordane; α-HCH and *p*,*p*′-DDD). CB 153 was the most abundant PCB congener in both body fluids (serum: 201 ± 106 pg/ml, follicular fluid: 72 ± 44 pg/ml). The average lipid content of serum samples was 5.04 ± 0.74 g/l. Compound concentrations were significantly different in serum and follicular fluid, with higher levels found in serum (Table II).

For every compound, strong correlations were detected between serum and follicular fluid concentrations ($r \geq 0.93$), except for HCB ($r = 0.631$; Figure 1). Moreover, for the compounds detected in nearly every follicular fluid and serum sample, linear regression models to predict follicular fluid EDC concentrations from serum were statistically significant (Figure 1). The same result was obtained using log-transformed values (data not shown). Age was positively correlated with nearly every compound in both the follicular fluid and serum, whereby CB 170 ($r = 0.628$) and oxychlordane ($r = 0.783$) showed the highest and β-HCH ($r = 0.424$) and CB 118 ($r = 0.468$) the lowest correlations with age in the follicular fluid and serum, respectively. No correlations were found in the follicular fluid between BMI and the detected chemical compounds. In serum samples, BMI showed moderate positive correlations with only two compounds: HCB ($r = 0.520$) and β-HCH ($r = 0.477$). The number of retrieved oocytes did not correlate with any compounds in both follicular fluid and serum. Low negative correlations were found between [E2] and several compounds in both body fluids ($-0.337 \leq r \leq -0.522$). A correlation table of the patient and cycle characteristics and the detected compounds in the follicular fluid and serum can be found in Supplementary data (Table SI and SII).

To test the relationship between the contamination status of the follicular fluid and the proportion of high-quality embryos, we first summarized the nine original variables into new summary values (PC scores) using PCA. Next, we found that the first PC score (PC 1) encompasses 64% of all the information contained in the nine original

Table II Mean concentrations with SD [pg/ml and ng/g lipid weight (lw)] and DF (%) of different endocrine-disrupting chemicals in serum and follicular fluid samples from women undergoing ART.

Compound	DF	Serum ($n = 20$)		DF	Follicular fluid ($n = 40$)
		pg/ml	ng/g lw		pg/ml
CB 105	75	8 ± 5	1.5 ± 0.9	0	
CB 118	100	42 ± 28[a]	8.1 ± 4.8	68	15 ± 8[b]
CB 138	100	120 ± 57[a]	23.5 ± 9.2	98	49 ± 32[b]
CB 153	100	201 ± 106[a]	39.2 ± 17.6	98	72 ± 44[b]
CB 170	100	44 ± 23[a]	8.7 ± 4.4	85	21 ± 13[b]
CB 180	100	111 ± 61[a]	21.9 ± 10.9	93	51 ± 33[b]
CB 183	100	12 ± 7	2.4 ± 1.1	5	13 ± 3
CB 187	100	25 ± 16	4.9 ± 2.8	30	18 ± 10
Sum PCBs		562 ± 294[a]	110.1 ± 48.9		213 ± 136[b]
HCB	100	59 ± 31[a]	11.3 ± 4.7	93	32 ± 19[b]
OxC	100	21 ± 11	4.0 ± 1.6	0	
TN	100	15 ± 10	2.9 ± 1.8	0	
p,*p*′-DDE	100	622 ± 406[a]	119.8 ± 68.8	98	392 ± 348[b]
p,*p*′-DDT	70	16 ± 12	3.4 ± 2.0	8	35 ± 5
β-HCH	95	35 ± 22[a]	6.9 ± 3.7	78	34 ± 35[b]
γ-HCH	45	7 ± 2		5	34 ± 1
BDE 47	83	3 ± 3	0.7 ± 0.7	3	12
BDE 99	5	2 ± 1	0.5 ± 0.1	3	14

LOQs ranged from 2 to 30 pg/ml and from 0.4 to 4 ng/g lw. Compounds with DF >50% were selected for statistical analysis.
DFs, detection frequencies; CB, chlorinated biphenyl; HCB, hexachlorobenzene; OxC, oxychlordane; TN, *trans*-nonachlor; DDE, dichlorodiphenyldichloroethylene; DDT, dichlorodiphenyltrichloroethane; HCH, hexachlorocyclohexane; BDE, brominated diphenyl ether.
[a,b]Data differ significantly among groups ($P < 0.05$).

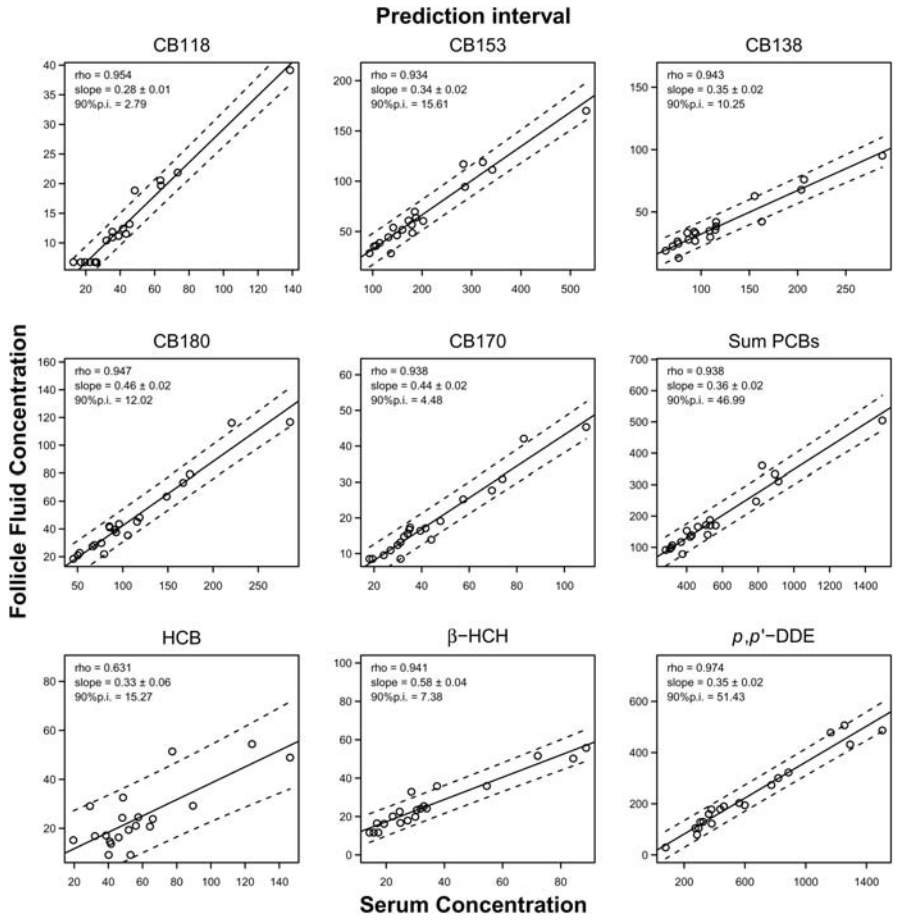

Figure 1 Linear regression models with serum as independent and follicular fluid concentrations as dependent variable. The solid line indicates the linear regression line. Dashed lines indicate the 90% prediction interval. For each of the compounds, the legend in the top left corner contains the Spearman correlation coefficient (rho, $P < 0.01$), the slope of the linear regression model and its standard error, and 90% prediction interval (90%pi, pg/ml) for the mean value for x.

variables, characterizing the follicular fluid contamination using the following formula: PC 1 = −0.296 × [CB 118] − 0.359 × [CB 153] − 0.380 × [CB 138] − 0.339 × [CB 180] − 0.339 × [CB 170] − 0.366 × [sum PCBs] − 0.326 × [HCB] − 0.162 × [β-HCH] − 0.378 × [*p*,*p*′-DDE]. This means that the lower the PC 1 score, the higher the overall contamination status of the follicular fluid in that patient, and that this PC1 score can be used as a summary statistic for the overall contamination status of the follicular fluid. Logistic regression models with fertilization rate, proportion of high-quality embryos relative to both the number of retrieved oocytes and 2PN-zygotes, ART- or pregnancy results as outcome, and age, [E2], BMI, fertilization procedure and male subfertility as explanatory variables showed a significantly improved fit upon adding PC1 as a covariate for fertilization rate ($P < 0.00001$) and the proportion of high-quality embryos relative to the number of retrieved oocytes ($P < 0.05$). This suggests that a lower PC1 score, meaning higher EDC concentrations in the follicular fluid, leads to a lower fertilization rate and subsequently, to a lower proportion high-quality embryos. These significances remain if only those patients are considered in which the original fertility problem was attributed to a male factor ($n = 27$). Furthermore, BMI was shown to have a significant effect on the proportion of high-quality embryos relative to both the number of retrieved oocytes ($P < 0.01$) and 2PN-zygotes ($P < 0.001$). In both cases, a higher BMI leads to a lower proportion of top-quality embryos. No associations were found between clinical pregnancy and live birth rate and the contamination status of follicular fluid. Full logistic regression results are shown in Supplementary data, Table SIII and the goodness of fit of the models with significant associations is shown in Supplementary data, Figure SI. The addition of PC2, which explains only 24% of the information of the nine original variables, to this logistic regression model did not improve the fit of the model ($P > 0.05$).

Discussion

In this study, we hypothesized that the presence of EDCs in the follicular micro-environment may be a risk factor for impaired oocyte development. Therefore, we aimed first to determine EDC concentrations in the follicular fluid of women undergoing ART procedure. Consequently, we related these concentrations to the patient's profile and ART characteristics. Finally, because follicular

fluid can only be obtained in clinical settings, we verified if EDC levels in the follicular fluid could be predicted based on serum measurements.

To our knowledge, our results are the first to report a significant *in vivo* association between higher EDC concentrations in the follicular fluid of a patient and reduced developmental competence of her oocytes which were directly exposed to these EDCs *in vivo*. Using PCA, we demonstrate that patients with lower PC1 scores, which reflect higher EDC contamination of the follicular fluid, have a highly significant drop in fertilization rate and a significant lower proportion of top-quality embryos, independent of the age, BMI, E2 levels of the patient, fertilization procedure or the presence of male subfertility. The strong influence of the follicular fluid contamination status on the fertilization rate is likely to be the most important factor to explain the observed lower proportion of high-quality embryos. It is clear that if fewer oocytes are fertilized, less can develop into high-quality embryos. This conclusion is supported by the observation that the proportion of 2PN-zygotes that develop into top-quality embryos is not affected by the PC1 score; in other words, if an oocyte has the potential to develop into a zygote, its chance to further develop into a high-quality embryo is not hampered by the EDC concentration in the follicular fluid. On the contrary, a higher BMI was shown to have a profound negative effect on the proportion of 2PN-zygotes that produce high-quality embryos. Because fertilization rate is not influenced by BMI and the BMI effect is most clear on the number of top-quality embryos relative to the amount of 2PN-zygotes, it could be speculated that BMI particularly impairs the first steps of embryonic development. Because embryo quality is an important determinant of successful implantation (Cakmak and Taylor, 2011), the observed lower proportion of high-quality embryos in our study, paralleled by decreased fertilization rates, may thus (partially) explain the lower implantation rates described recently in women with higher PCB concentrations in their serum (Meeker *et al.*, 2011), which however could not be confirmed by our data. Until our study, the only available information about follicular fluid contamination with EDCs and IVF outcome was the suggestion of a possible link between the presence of *p,p′*-DDE in the follicular fluid and serum and an impaired fertilization rate (Younglai *et al.*, 2002; Weiss *et al.*, 2006). Our study significantly confirms this negative effect of EDC contamination in the follicular fluid on fertilization rates. However, cleavage rate seems not to be affected by the presence of EDCs (Jarrell *et al.*, 1993). In a Czech study, only non-significant trends were found between PCB levels in the follicular fluid and ART results. However, they did not measure the most frequently detected PCB congeners (CB 138, CB 153 and CB 180) and so certain associations were maybe underestimated (Jirsova *et al.*, 2010). In our study, no associations were found between ART- and pregnancy outcome and the EDC contamination status of follicular fluid. This can be explained by the fact that although fertilization rate and embryo quality are imperative factors, there are undoubtedly other aspects later in embryonic development (e.g. uterine environment) which are also essential for the establishment of a successful pregnancy (Singh *et al.*, 2011).

In antral follicles, oocytes and follicular somatic cells are in close contact with the EDCs present in the follicular fluid, which is why EDCs can directly interact with these cells and influence the processes of folliculo- and/or oögenesis with a potential diminished oocyte quality as a result. A substantial amount of *in vitro* studies already reported on the inability of oocytes to fulfill both nuclear and cytoplasmic maturation after exposure to generally high EDC concentrations during *in vitro* maturation (Campagna *et al.*, 2001; Pocar *et al.*, 2001; Lenie *et al.*, 2008). Furthermore, oocytes that were exposed to a PCB mixture, which approaches the PCB levels in the follicular fluid of our study, were able to successfully complete maturation, while a significant number of them failed to reach the blastocyst stage (Pocar *et al.*, 2001). This observation underpins the finding that the initial quality of the oocyte is not only imperative for its own maturation, but that it is also fundamental for its further developmental competence (Sirard *et al.*, 2006). Also, cumulus and granulosa cells were vulnerable to the action of EDCs (Campagna *et al.*, 2001; Younglai *et al.*, 2004; Mlynarczuk *et al.*, 2009; Kwintkiewicz *et al.*, 2010). Although we recognize the possible existence of other confounders which were not taken into account in our study, such as the smoking habits of the parents (Cooper and Moley, 2008; Calogero *et al.*, 2009), the presence of undefined compounds in the follicular fluid, the metabolic state of the mother (Van Hoeck *et al.*, 2011) or the lack of paternal exposure data, we point out that our *in vivo* study indicates that the harmful influence of relatively low EDC concentrations on embryo development observed *in vitro* indeed characterizes what can happen *in vivo*. Another possible source of bias might be the choice of fertilization procedure. However, assignment of patients to ICSI was decided based on the local clinical criteria of the participating hospital before PCB exposure was known. There was no evidence of an association between male subfertility and fertilization procedure. The use of varying criteria between hospitals and the relatively small sample size preclude investigation of whether the effect of PCBs on fertilization differed between IVF and ICSI.

This research also contributes to the crucial global monitoring of EDCs in the follicular fluid (Caserta *et al.*, 2011). Our results confirm the worldwide declining trend of well-known EDC levels in the follicular fluid (Trapp *et al.*, 1984; Baukloh *et al.*, 1985; Schlebusch *et al.*, 1989; Jarrell *et al.*, 1993; Pauwels *et al.*, 1999; Younglai *et al.*, 2002; De Felip *et al.*, 2004; Weiss *et al.*, 2006; Meeker *et al.*, 2009; Jirsova *et al.*, 2010). More specifically, the concentration of CB 153 (the most abundant PCB congener) in our study is more than two times lower than the studies executed around the turn of the century (Pauwels *et al.*, 1999; Younglai *et al.*, 2002; De Felip *et al.*, 2004) and comparable with CB 153 levels in a more recent report (Meeker *et al.*, 2009). In the Czech study, the total PCB concentration was more than four times lower, which can again be explained by the fact that they studied rather rarely detected PCB congeners (Jirsova *et al.*, 2010). HCB levels dropped considerably as compared with the first measurement of pollutants in the follicular fluid (Trapp *et al.*, 1984) with a tendency for stabilization in the last decade (De Felip *et al.*, 2004; Meeker *et al.*, 2009). The levels of *p,p′*-DDE are more variable between countries which can be caused by a different use of DDT in the past (Jarrell *et al.*, 1993; De Felip *et al.*, 2004; Weiss *et al.*, 2006; Meeker *et al.*, 2009). Nonetheless BDE 47 and BDE 49 were only detected in one sample, to the best of our knowledge, it is the first time that PBDE congeners are detected in the follicular fluid. In case of our serum samples, EDC concentrations are in the same order of magnitude with recent studies in other Western countries (Porta *et al.*, 2010; Herrick *et al.*, 2011; Kalantzi *et al.*, 2011), with a distinct decline in contamination levels compared with

older studies (Pauwels *et al.*, 1999; Voorspoels *et al.*, 2002; Needham *et al.*, 2005). Also, the bioaccumulation potential of these EDCs is once more demonstrated by the positive correlation between the age of the patients and the contamination levels in both serum and follicular fluid.

Based on our results, we also confirm that for compounds, regularly detected in both serum and follicular fluid samples (Pauwels *et al.*, 1999; Younglai *et al.*, 2002; Meeker *et al.*, 2009), the screening of serum is an easy and reliable approach to predict EDC contamination of follicular fluid, as was previously suggested (Pauwels *et al.*, 1999; Meeker *et al.*, 2009). This can be largely attributed to the very high correlations between the contaminant concentrations in follicular fluid and serum observed in every study except for the study of Jarrell *et al.* (1993). This inconsistency can be explained by the rather high limits of detection in the latter study which resulted in a substantial number of 'blank' samples, which implicitly may have influenced statistical analysis. On the contrary, the correlations calculated in our study are similar to the ones in a previous Belgian study (Pauwels *et al.*, 1999) and slightly higher than in a US study (Meeker *et al.*, 2009). Roughly, concentrations of PCB congeners, *p,p′*-DDE and HCB were around two to three times higher in serum than in follicular fluid and in the middle of the range described in other studies (Jarrell *et al.*, 1993; Pauwels *et al.*, 1999; Weiss *et al.*, 2006; Meeker *et al.*, 2009). Because the vast majority of EDCs are lipophilic substances, the fact that serum contains more lipids than follicular fluid is the main reason for the difference in the contamination status of both body fluids (De Felip *et al.*, 2004; Browne *et al.*, 2008). To improve precision, regression models were built allowing to predict with 90% accuracy the follicular fluid EDC contamination—and thus the direct *in vivo* exposure of human oocytes and follicular somatic cells to EDCs—when only serum samples are available. Because our patient group shares several characteristics like weight, height and BMI with pregnant volunteers from larger biomonitoring studies and the serum levels of EDCs from these volunteers are in the same range as those in our study (Koppen *et al.*, 2009; Darnerud *et al.*, 2010; Llop *et al.*, 2010), we are confident that our study group matches the profile of the Western (pregnant) female population in terms of EDC contamination. Keeping this in mind and given the strong correlations between serum and follicular fluid concentrations of the most frequently detected EDCs, and because it is ethically impossible to obtain follicular fluid samples from women who are not undergoing ART, we recommend our regression models as useful tools to estimate the EDC contamination of the follicular micro-environment of these women outside an ART setting.

In conclusion, our study is the first to document overall higher EDC contamination in the follicular micro-environment being associated with a lower chance of an oocyte to develop into a top-quality embryo, mainly due to a reduced fertilization rate. Further research is needed to explore this interesting link. Also, this study gives an update on and contributes to the essential continuous monitoring of the contamination status of follicular fluid with well-known persistent EDCs. In general, the levels of these substances are declining and stabilizing to background concentrations, a finding which puts forward the need to further investigate the presence of some other 'newly' emerging contaminants in the follicular micro-environment. PBDE levels are detected for the first time in the follicular fluid, however only in one sample. Serum levels of EDCs are comparable with other recent studies. Furthermore, the strong correlations between serum and follicular fluid EDC concentrations, enabled us to build, for the most detected compounds, linear regression models that predict with 90% accuracy the follicular fluid concentrations of EDCs based on serum levels. In this way, follicular fluid samples are not crucial anymore to gain knowledge about the contamination status of the follicular micro-environment with these EDCs.

Supplementary data

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

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Authors' roles

E.M.L.P. executed and coordinated the study, was responsible for submitting the essential documents to the ethical committee of the University of Antwerp and wrote het manuscript. J.L.M.R.L. provided critical input on the study design and planning. A.C. and A.C.D. both specialized in chemical analysis, assisted with the GC/MS analyses. E.F. provided critical input on and executed the statistical analysis. D.N. was responsible for the study coordination at and for submitting the essential documents to the ethical committee of the ZNA Middelheim hospital. I.P. was responsible for serum and follicular fluid sampling. P.E.J.B., supervisor of E.M.L.P. gave critical assistance in the study design and planning. All co-authors reviewed and approved the final manuscript.

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Conflict of interest

None declared.

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