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# Persistent organic pollutants (POPs) in human follicular fluid and in vitro fertilization outcomes, a pilot study

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ABSTRACT

Persistent organic pollutants (POPs) are ubiquitously distributed among the U.S. population and adversely impact human reproduction. These compounds have been detected in human ovarian follicular fluid (FF), where they directly contact a developing oocyte. As a pilot investigation, we measured 43 polychlorinated biphenyl (PCB) congeners, p,p'-dichlorodiphenyltrichloroethane (DDT), and its persistent metabolite p,p'-dichlorodiphenyldichloroethylene (DDE) in residual FF collected from 32 women undergoing in vitro fertilization (IVF). We identified significant inverse associations between higher levels of PCB congeners and indicators of ovarian reserve (e.g., antral follicle count), follicular response to administered gonadotropins (e.g., peak estradiol, number of oocytes retrieved, endometrial thickness), intermediate IVF endpoints (e.g., oocyte fertilization and embryo quality), and clinical IVF outcomes (e.g., embryo implantation and live birth), after adjusting for body mass index, cigarette smoking, race, and age. Our results suggest that ongoing exposure to POPs impacts IVF and merit confirmation in a larger and more definitive future study.



## 1. Introduction

Nearly four million U.S. women desired, but were unable to conceive children in 2002, according to data from the National Survey of Family Growth [1]. Between 1982 and 2002 the number of women reporting difficulty conceiving and maintaining pregnancy increased by about 60%, and 200% for women less than 25 years old [2], suggesting important causal factors in addition to advancing average maternal age [3]. Clinicians and investigators alike have expressed growing concern that exposure to environmental toxicants contributes to infertility [4].

Environmental endocrine disrupting compounds, including polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs), are widely reported to interfere with reproductive function in vitro, in experimental animal models, and in epidemiologic human studies [5]. These persistent organic pollutants (POPs) were extensively employed for myriad industrial and consumer applications, although concerns about their toxicity and propensity to bioaccumulate in lipids led to global restrictions in their use and manufacture over the past few decades [6,7]. Consequently, exposures to POPs have dropped in recent years, yet ongoing contamination of food and water sources continue to foster non-occupational, or 'background' exposures in general populations [8,9], including among reproductive-aged women [10,11].

PCBs comprise a family of 209 related structures, or congeners, distinguished by the pattern of chlorine substituents on a biphenyl ring [12]. The distribution of chlorine substituents governs biologic activity, including estrogen receptor binding [13]. Heavier pentachlorinated to octachlorinated PCB congeners are highly persistent in vivo, often with human half-lives measured in years, and tend to accumulate over time, whereas less chlorinated congeners are more likely to be metabolized and excreted [14,15]. Given their use as various congener mixtures and their similar physical properties, PCB congeners tend to migrate together in environmental compartments, although transformation and differ-

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ential accumulation make isolation of individual biologic effects challenging [16,17].

PCBs and OCPs have been detected in human ovarian follicular fluid (FF) [18–26], underscoring the proximity of these agents to a developing oocyte, and their potential to impact women's reproductive health [27]. Higher exposure levels are linked to decreased fecundity, or reproductive capacity, among women conceiving spontaneously [28–31] and also among those using in vitro fertilization (IVF) [20,21,32,33]. However, the evidence to date is controversial [24,34–37], prompting calls for additional investigation [38].

To further investigate the impact of POPs on human reproduction, we conducted a pilot study of PCBs and OCPs in FF specimens collected from women undergoing IVF at an academic reproductive health clinic. The aim of this hypothesis-generating pilot study was to characterize the distribution of POPs exposure in FF, and to explore their associations with IVF endpoints. Positive findings from this work should be confirmed in a larger future study.

## 2. Material and methods

### 2.1. Study sample

Fifty eight female patients undergoing a 1st completed IVF cycle at the University of California at San Francisco Center for Reproductive Health (USA) were recruited to participate in the Study of Metals and Assisted Reproductive Technologies (SMART) between 3/12/07 and 4/29/08 [39]. Patients referred to the clinic for infertility treatment received an initial evaluation comprised of gynecologic, reproductive, and medical histories. A physical examination was performed that included a transvaginal ultrasound to evaluate the pelvic organs. A blood specimen was collected to assess reproductive hormone levels. A hysterosalpingogram or saline hysterosonogram was usually performed to assess tubal patency and uterine cavity contour. Male partners received an initial reproductive evaluation that specifically focused on semen quality analysis to assess volume, number, motility, and morphology. Patients and their male partners completed an infertility history questionnaire to ascertain demographic factors including age, race and ethnicity, as well as health related behaviors, including cigarette smoking and alcohol use. Following the baseline evaluation or pre-cycle consultation, patients underwent gonadotropin-induced ovarian follicle stimulation according to clinic protocols. Ultrasounds and daily serum estradiol ($E_2$) measures were performed to assess and confirm uterine lining development and ovarian follicle maturation. Patients' responses were 'mapped' on a stimulation sheet with sizes and numbers of follicles delineated during stimulation. When a sufficient number of follicles had developed to >16 mm diameter approximately two weeks later, human chorionic gonadotropin (hCG) was administered, and oocytes were retrieved using fine needle aspiration within 34–36 h.

We described the clinical IVF protocol in detail in a previous publication [39]. In brief, on the day of oocyte retrieval, ovarian follicles were evacuated using a transvaginal ultrasound probe with an 18 gauge, 36 cm aspiration needle. Oocytes isolated from FF were fertilized with sperm collected from the male partner on the day of oocyte retrieval, using conventional fertilization or intracytoplasmic sperm injection (ICSI). Oocytes for ICSI cases were denuded of cumulus cells and examined for the presence of a single polar body indicative of metaphase-2 (MII) arrest prior to insemination. For conventional cases, oocytes were incubated with sperm and then examined for the presence of polar bodies 24 h later. Oocytes from all cases were examined for the appearance of two pronuclei (2PN) indicative of normal fertilization approximately 24 h after insemination. Following fertilization, cultured embryos were evaluated for embryo fragmentation score (a negative predictor of implantation (EFS)) [40] and embryo cell number (a positive predictor of implantation (ECN)) [41] by embryologists blinded to all exposure measurements and using ordinal scales. EFS and ECN were assessed on the day of transfer; on day 3 in a majority of cases as day 2 assessment was made only for a limited cohort of embryos. A standardized morphological embryo grading system has been established within our IVF laboratory. Based on clinical experience we dichotomized embryo quality as 'good,' for embryos with ECN $\geq$ 6 and EFS $\leq$ 2, and 'poor' for embryos with ECN <6 and EFS >2, as these scores were predictive of outcomes in our lab. Fertilized embryos were transferred to patients on day 2–3 post-fertilization contingent on clinical factors. Serum hCG tests two weeks later indicated embryo implantation. Patients were contacted nine months later to obtain live birth outcomes.

### 2.2. Laboratory analysis

The 0.5–5 mL of FF isolated from each ovarian follicle during oocyte retrieval is usually discarded as medical waste. We retained and pooled FF from the largest follicle in each ovary for n = 46 women, which was spun for 10 min at 1500 g, and then aliquoted into 1.8 mL cryovials prior to storage at −80 °C. No media was used in FF tubes and we excluded 'flushing media' from the analytic specimen. FF specimens were shipped on dry ice to the Wadsworth Center, New York State (NYS) Department of Health (Albany, NY USA), where they were initially analyzed for trace elements by the Laboratory of Inorganic and Nuclear Chemistry as previously reported [42]. Residual FF specimens for 32 women were analyzed by the Wadsworth Center Laboratory of Organic Analytical Chemistry.

We measured POPs in FF using modifications to a method previously optimized for quantifying ultratrace levels of PCBs in human sera [43]. We determined concentrations for 43 PCB congeners as numbered by the International Union of Pure and Applied Chemistry (IUPAC #'s 28, 44, 49, 52, 66, 74, 77, 81, 87, 99, 101, 105, 110, 114, 118, 123, 126, 128, 138, 146, 149, 151, 153, 156, 157, 158, 167, 169, 170, 172, 177, 178, 180, 183, 187, 189, 194, 195, 196–203, 199, 201, 206, 209), p,p'-dichlorodiphenyltrichloroethane (DDT), and the highly persistent DDT metabolite dichlorodiphenyldichloroethylene (DDE). Approximately 1 mL aliquots of FF were spiked with isotopically-labeled internal standards (Cambridge Isotope Laboratories, Inc. Tewksbury, MA USA) $^{13}$C PCB IUPAC #'s 28 (for trichlorobiphenyls), 52 (for tetrachlorobiphenyls), 118 (for pentachlorobiphenyls), 153 (for hexachlorobiphenyls), 180 (for heptachlorobiphenyls), 194 (for octachlorobiphenyls), 206 (for nonachlorobiphenyls), 209 (for decachlorobiphneyls), and $^{13}$C DDE (for OCPs). Samples were extracted using a Biotage automated solid phase extraction system (Charlotte, NC USA), and then analyzed by gas chromatography with mass spectrometry (GC/MS) using an Agilent Technologies 6890 gas chromatographer (Santa Clara, CA USA) coupled to an Agilent 5975C mass spectrometry detector in selected ion monitoring mode. We implemented standard laboratory quality assurance/quality control protocols for organic analytes during the analysis, including a five point matrix-matched calibration, a method detection limit (MDL) determination, and an instrument detection limit study. Method and reagent blanks, a laboratory fortified sample matrix, a continuing calibration verification standard, and a second source standard were run with each batch of FF specimens. All solvents and reagents were lot checked prior to use. Microliter syringes and volumetric glassware are calibrated semi-annually and analytical balances are certified annually by All-State Scale Co. (Somers, CT USA).