Endocrine disruptors and human reproductive failure: the in vitro effect of phthalates on human luteal cells

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Objective: To evaluate the influence of phthalates on human luteal cell function.
Design: Laboratory study.
Setting: University hospital.
Patient(s): Twenty-three normally menstruating patients in the midluteal phase.
Intervention(s): Human luteal cells isolated from corpora lutea for primary cultures.
Main Outcome Measure(s): Progesterone (P4) and prostaglandin release assayed by enzyme immunoassay, vascular endothelial growth factor (VEGF) secretion by enzyme-linked immunosorbent assay (ELISA), and VEGF mRNA expression by real-time polymerase chain reaction.
Result(s): We investigated the effect of di(2-ethylhexyl)phthalate (DEHP), di-n-butyl phthalate (DBP), and butyl benzyl phthalate (BBP) on basal and hCG-induced progesterone (P4) release, as well as DEHP effect on the balance between prostaglandin (PG) E2, vascular endothelial growth factor (VEGF)-luteotrophic factors, and the luteolitic PGF2α in isolated human steroidogenc cells. Phthalates influence on VEGF expression has been also evaluated. DEHP, DBP, and BBP were able to reduce both basal and hCG-stimulated P4 as well as PGE2 release. PGF2α release was reduced after DEHP incubation. VEGF protein release was decreased by the incubation with the tested phthalates. VEGF mRNA expression was not affected by DEHP, DBP, and BBP. As expected, both hCG and cobalt chloride were able to induce P4 release and VEGF release and mRNA expression in human luteal cells respectively.
Conclusion(s): The results show the ability of phthalates to affect luteal steroidogenesis as well as the balance between luteotrophic and luteolytic factors suggesting an interference of phthalates in human luteal function. These data may contribute to clarify the classically known impaired reproductive health observed after phthalates exposure. (Fertil Steril® 2014;102:831–7. ©2014 by American Society for Reproductive Medicine.)
Key Words: Corpus luteum, endocrine disruptors, luteal function

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Phthalates are synthetic chemicals that belong to the endocrine disruptor family (1), and their high production volume has resulted in ubiquitous human exposure (2). Globally, more than 18 billion pounds of phthalates are used each year, primarily as plasticizers in flexible polyvinyl chloride (PVC) products (2, 3); used to impart flexibility to plastics, they can be found in most PVC products including vinyl upholstery, tablecloths, shower curtains, several sprays including pesticides, solvents, and soft-squeeze children's toys. The use of these compounds also is approved in several medical devices such as tubing, blood bags, and vinyl gloves, and they also can be found as additives in cosmetics products such as lotion, perfume, and nail polish.

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The high production volume and common use of phthalates has resulted in humans commonly being exposed to phthalates on a daily basis through ingestion, inhalation, and dermal contact (4–6). Because these plasticizers cannot form strong molecular bonds with polymers, they are rapidly leached into the environment and have become a widespread environmental contaminate (2, 7–10).

Dozens of phthalates have been developed over the years, and an association between phthalate exposure and adverse reproductive health outcomes has been suggested (11). Interestingly, phthalates have been demonstrated to reduce fertility in both humans and animal models (12), and occupational exposure to phthalates has been associated with decreased pregnancy rates, increased miscarriages, and other gestational complications (13, 14). Moreover, it has been demonstrated that these plasticizers negatively modulate oocyte growth, ovulation, and embryonic development, leading to impaired ovarian function (12, 15). Interference with ovarian steroid synthesis and metabolism could partially explain these effects. Indeed, phthalates exert antiandrogenic or estrogenic effects in vitro, impairing 17β-estradiol affinity to its receptors (2, 16–18) and modulating steroid biosynthesis and metabolism by acting as a peroxisome proliferator-activated receptor (PPAR) agonist as well (11).

Of note, among the phthalates, di-(2-ethylhexyl) phthalate (DEHP) is well known to be the most abundant and potent reproductive toxicant chemical (Center for the Evaluation of Risks to Human Reproduction-CERHR 2000). Nevertheless, women of reproductive age have a specific exposure risk profile to di-*n*-butyl phthalate (DBP), which is commonly used in many beauty products (2, 19). Finally, exposure to *n*-butyl benzyl phthalate (BBP) is well known to be associated with reproductive alterations in males as well as with gynecologic diseases such as endometriosis (20, 21).

To expand the available data on impaired reproductive-hormone balance resulting from phthalates exposure, our in vitro study investigated the potential effects of DEHP, DBP, and BBP on human luteal cell function. The corpus luteum is a unique transient reproductive gland essential for the establishment and maintenance of early pregnancy. In particular, corpus luteum function is a hormone-dependent phenomenon. Both alpha and beta estrogen receptors as well as PPAR are expressed in the corpus luteum. Through these receptors, xenoestrogens such as phthalates can exert their influence on luteal function. We investigated whether these plasticizers can affect both basal and gonadotropin-stimulated luteal progesterone (P4) production in highly purified human luteal cells. Moreover, to evaluate the possible alteration of the balance between intraovarian luteotrophic and luteolytic regulators, we analyzed the effect of DEHP, DBP, and BBP on the luteal release of vascular endothelial growth factor (VEGF) and prostaglandin E2 (PGE2)—both local luteotrophic factors (19, 20)—and PGF2α, a classic luteolytic modulator (22, 23). In addition, we examined the effect of DEHP, DBP, and BBP in modulating VEGF luteal mRNA expression.

MATERIALS AND METHODS

Cell Cultures

Corpora lutea were obtained from 23 normally menstruating women (25 to 38 years old) in the midluteal phase (days 5 to 6 from ovulation) at the time of surgery for nonendocrine gynecologic diseases. The protocol was approved by the institutional review board of Università Cattolica del Sacro Cuore in Rome, and all patients provided written informed consent. The corpora lutea were dated on the basis of the presumptive day of ovulation (day 0), as determined by urinary luteinizing hormone (LH) peak, ultrasound detection of corpus luteum or disappearance of the dominant follicle, and a rise in the plasma P4 concentration.

The human luteal cells cultures were performed as previously reported elsewhere (24). Briefly, luteal tissue was enzymatically dissociated in nutrient mixture F-12 (Flow Laboratories)/Hepes (Sigma-Aldrich) medium containing type IV collagenase (200 U/mL) (Sigma-Aldrich). The identify of both large and small human luteal cells was confirmed by their positive staining for lipids with oil red O14 (25). Isolated human luteal cells (250,000 cells/mL) were plated on 48-well dishes for enzyme-linked immunosorbent assay (ELISA) or enzyme immuno assay (EIA) or for real-time polymerase chain reaction (PCR) and cultured for 24 hours in 5% CO_2 and 95% air at 37°C.

At the end of isolation and 24 hours after all the treatments, human luteal cells were counted in a hemocytometer, and their viability was determined by trypan blue exclusion test. Neither treatment nor solvent for dissolving tested substances affected either the cell count or cell viability.

Human luteal cells were incubated for 24 hours with serum-free medium alone (control), human chorionic gonadotropin (hCG, 100 ng/mL; Calbiochem Inalco), cobalt(II) chloride ($CoCl_2$, 10 μM, chemical hypoxia), DEHP (10^{-6}–10^{-9} M), DBP (10^{-6}–10^{-9} M), or BBP (10^{-6}–10^{-9} M) (Sigma Aldrich s.r.l.) alone or with hCG (100 ng/mL). Three different wells were used for each experimental condition. The doses we used of the phenols were not greater than the previously demonstrated serum and follicular fluid levels (26, 27). After the incubation, human luteal cells were treated for total RNA extraction while the culture media were separately collected and assayed for P4, PG, and VEGF detection.

PGs, P4, and VEGF Assays

According to manufacturer's instructions, the PGE2, PGF2α, and P4 levels in the culture media were assayed with specific EIA kits (Cayman Chemical) and VEGF with a VEGF ELISA kit (R&D Systems). For P4, the EIA kit intra-assay coefficient of variation (CVw) and interassay coefficient of variation (CVb) were 7% and 9%, respectively, and the detection limit (DL) was 10 pg/mL. For PG, the EIA kit CVw and CVb were 12% and 9%, respectively, and the DL was 9 pg/mL. For VEGF, the ELISA kit CVw and CVb were 3.5% and 6.7%, respectively, and the sensitivity was 5.0 pg/mL.