Follicle growth is inhibited by benzo-[α]-pyrene, at concentrations representative of human exposure, in an isolated rat follicle culture assay

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BACKGROUND: The adverse effects of cigarette smoking on human fertility have been well documented. However, the mechanism(s) underlying the detrimental effects of cigarette smoking are unknown. Using a novel isolated rat follicle culture assay, we tested the hypothesis that benzo-[α]-pyrene (B[α]P), a constituent of cigarette smoke, can inhibit follicle growth. METHODS: B[α]P levels were quantified in the serum and follicular fluid (FF) of women undergoing in vitro fertilization (IVF) treatment exposed to mainstream smoke (n = 19) and non-smokers (n = 10) by gas chromatography mass spectrometry. Isolated rat follicles were cultured with increasing concentrations of B[α]P (1.5–300 ng ml⁻¹) and follicle diameter was measured daily. RESULTS: Mean (±Standard error of the mean) B[α]P was quantified in the serum (0.40 ± 0.13 ng ml⁻¹) and FF (1.32 ± 0.68 ng ml⁻¹) of women who smoke. IVF stimulation and outcome measures were similar between female smokers and non-smokers with the exception of implantation rate and pregnancy rate, which were both significantly lower (P < 0.05) in the MS group. B[α]P treatment significantly reduced rat follicle diameter and attenuated FSH stimulated growth in a dose-dependent manner, beginning at 1.5 ng ml⁻¹. CONCLUSIONS: Our data suggest that B[α]P, at levels representative of those measured in human FF, may adversely affect follicle development and be an ovarian toxicant.

Key words: benzo-[α]-pyrene/cigarette smoke/fertility/folliculogenesis

Introduction

Epidemiological studies demonstrate that female smokers have delayed conception (Jick and Porter, 1977), premature ovarian failure leading to an earlier onset of menopause (Jick and Porter, 1977; Howe et al., 1985; Baird et al., 2005) and lower success rates when using assisted reproductive technologies (Pattinson et al., 1991; Neal et al., 2003; Seltzer, 2003) compared with their non-smoking (NS) counterparts. Compelling evidence demonstrates that exposure to cigarette smoke can affect ovarian function (Zenzes et al., 1995). Furthermore, we have recently shown that side-stream smoke exposure is equally as damaging to a woman’s fertility as if she herself was a current smoker (Neal et al., 2005b). However, the toxic agents present in cigarette smoke, and the mechanisms of action responsible for the adverse effects of cigarette smoke on ovarian function have yet to be determined.

Isolating the chemical(s) in tobacco smoke responsible for altering fertility and accelerating menopause is complicated by the fact that cigarette smoke is composed of approximately 4000 chemicals, including nicotine, nitroso compounds, aromatic amines, protein pyrolysates and polycyclic aromatic hydrocarbons (PAHs) (Rustemeier et al., 2002; Lodovici et al., 2004). Only a few of these have been studied for their effects on the reproductive system (Zenzes, 2000). Of the PAHs present in cigarette smoke, we are interested in benzo-[α]-pyrene (B[α]P) because it has been shown to be present at high levels in cigarette smoke (Lodovici et al., 2004). The PAH family of chemicals are ubiquitous environmental toxicants present in air pollution, petroleum products and the diet in addition to occurring as a toxic bi-product of incomplete combustion from cigarette smoke. PAHs are known to exert their toxic effect through the aryl hydrocarbon receptor (Ahr) that is abundantly expressed in oocytes (Sun et al., 2004). However, the exposure levels of B[α]P, a constituent of cigarette smoke with a short half-life, in the serum and follicular fluid (FF) of reproductive aged women is unknown. Recent improvements among different in vitro follicle culture systems has provided a tool to investigate the effects of toxicants on follicle development from the primordial to antral stage (O’Brien et al., 2003; Sun et al., 2004). Therefore, the
objectives of the present study are to (i) quantify the levels of B[a]P in human serum and FF of smoking and NS women undergoing in vitro fertilization (IVF) treatment and (ii) determine the effect of B[a]P, a contaminant present in cigarette smoke on follicle growth using an isolated rat follicle culture (IRFC) system.

Materials and methods

Materials

Culture media and reagents were purchased from Gibco BRL (Burlington ON, Canada). Agarose, collagenase (type 1A), DNase I, ITS, rat serum and ascorbic acid were obtained from Sigma-Aldrich Chemical Company (St Louis, MO, USA). Falcon culture dishes and 96-well plates were obtained from Becton-Dickinson (Mississauga, ON, Canada).

Quantification of benzo-[a]-pyrene in serum and follicular fluid of women

Patients undergoing IVF treatment were recruited to participate in the study. Signed consent was obtained from 29 study subjects according to the Hamilton Health Sciences Research Ethics Board and the Centre for Reproductive Care Research committee. Study subjects were classified on the basis of their self-reported smoking habit, into one of two groups (i) Mainstream smoke (MS; n = 19) or (ii) NS (n = 10). MS smoke was defined as the smoke that is inhaled by the smoker, and NS is someone not exposed to cigarette smoke. The average age of study subjects in both groups was <35 years (Table I) and ranged between 26 and 37 for MS group when compared with 27–37 for the NS group. Diagnoses included male factor, tubal occlusion, combined male and female factors, endometriosis, PCOS and idiopathic infertility. Reasons for infertility were similar for both groups (Table II).

One blood sample was collected during the follicular phase (Days 5–7) of the IVF stimulation cycle, and FF was collected from the first aspirated follicle at the time of oocyte retrieval to obviate potential interference from contamination with blood in subsequent follicles. Samples were stored frozen (−80°C) until required for analysis. PAHs were extracted from serum and FF using dichloromethane. Extract clean-up was performed on 6% water deactivated aluminum oxide columns with hexane as the eluting solvent. The processed samples were analysed for B[a]P (detection limit = 5 pg ml⁻¹) and other PAHs using an Agilent 6890 gas chromatograph linked with Agilent 5973 N mass spectrometer (GC/MS) (Agilent Technologies, Palo Alto, CA, USA).

IVF stimulation and outcomes

All female study participants received s.c. injections of gonadotrophin-releasing hormone analogue, leuprolide acetate (Lupron, Abbott, Toronto, ON, Canada) 1 mg daily, from day 21 of the previous menstrual cycle for pituitary down-regulation. Recombinant follicle stimulating hormone (rFSH) (Gonal F, Serono, Oakville, ON, Canada) was used for all patients in this study and was initiated after the onset of menses. Ovarian suppression was confirmed by a serum estradiol (E2) of <150 pmol ml⁻¹ and the absence of ovarian cysts observed upon ultrasound examination. Dosage of rFSH was adjusted on the basis of the rise in E2 levels measured by radioimmunoassay, and the numbers of growing follicles were tracked by transvaginal ultrasound. When at least three follicles were ≥18 mm in diameter, 10 000 IU of human chorionic gonadotrophin (hCG) (Profasi; Serono, Oakville, ON, Canada) was administered i.m. Oocytes were retrieved using transvaginal ultrasound-guided aspiration 34–36 h after the hCG injection. Semen samples were provided by the male partner and processed by standard swim-up procedures. Insemination for conventional IVF or intracytoplasmic sperm injection (ICSI) was performed 4–6 h after oocyte retrieval. Oocytes and embryos were cultured in 60 μl drops

<table>
<thead>
<tr>
<th>Category</th>
<th>Non-smoking (NS)ᵃ</th>
<th>Mainstream smoking (MS)ᵃ</th>
<th>Statistics (P-values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Day 3 FSH</td>
<td>5.42 ± 0.88</td>
<td>6.18 ± 0.59</td>
<td>0.49</td>
</tr>
<tr>
<td>Age</td>
<td>34.10 ± 1.13</td>
<td>33.43 ± 1.10</td>
<td>0.69</td>
</tr>
<tr>
<td>Number of amps used</td>
<td>32.80 ± 3.46</td>
<td>29.54 ± 4.79</td>
<td>0.62</td>
</tr>
<tr>
<td>Estradiol at hCG</td>
<td>7 656.50 ± 944.63</td>
<td>6 740.00 ± 693.71</td>
<td>0.44</td>
</tr>
<tr>
<td>Number of oocytes retrieved</td>
<td>11.40 ± 1.09</td>
<td>9.56 ± 1.31</td>
<td>0.34</td>
</tr>
<tr>
<td>% mature (ICSI)</td>
<td>87.5 ± 1.25</td>
<td>78.7 ± 6.42</td>
<td>0.44</td>
</tr>
<tr>
<td>Fertilization rate</td>
<td>67.6 ± 8.31</td>
<td>54.9 ± 7.00</td>
<td>0.26</td>
</tr>
<tr>
<td>ET</td>
<td>2.40 ± 0.22</td>
<td>1.92 ± 0.24</td>
<td>0.21</td>
</tr>
<tr>
<td>FZ</td>
<td>2.10 ± 0.52</td>
<td>1.63 ± 0.68</td>
<td>0.54</td>
</tr>
<tr>
<td>48 h CES</td>
<td>12.00 ± 1.14</td>
<td>11.21 ± 0.91</td>
<td>0.59</td>
</tr>
<tr>
<td>72 h CES</td>
<td>22.55 ± 1.82</td>
<td>21.50 ± 1.82</td>
<td>0.79</td>
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<tr>
<td>ET CES</td>
<td>27.62 ± 1.74</td>
<td>25.38 ± 1.95</td>
<td>0.41</td>
</tr>
<tr>
<td>AUC</td>
<td>17 069.00 ± 2 842.32</td>
<td>19 978.56 ± 3 641.31</td>
<td>0.02</td>
</tr>
<tr>
<td>Utilization rateᵇ</td>
<td>62.1 ± 8.4</td>
<td>58.8 ± 5.7</td>
<td>0.74</td>
</tr>
<tr>
<td>Implantation rate</td>
<td>25.0%</td>
<td>12.0%</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Pregnancy rate</td>
<td>40.0%</td>
<td>11.1%</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

ICSI, intracytoplasmic sperm injection; ET, number of embryos transferred; FZ, number of embryos frozen; CES, cumulative embryo score; AUC, area under the curve.

ᵃValues are expressed as mean ± standard error of the mean.

ᵇUtilization rate is the sum of fresh transferred and frozen embryos divided by the number of zygotes.
of human tubal fluid medium (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% synthetic serum substitute (Irvine Scientific). Fertilization was determined by the presence of two pronuclei 16–18 h after insemination or ICSI. Fertilization rate was calculated as the number of zygotes divided by the total number of oocytes retrieved for each patient. Embryo utilization rate was used to determine embryo wastage and was calculated as the sum of fresh transferred and frozen embryos divided by the number of zygotes produced.

The stimulation parameters, number of oocytes retrieved, fertilization, meiotic maturity based on the presence of the first polar body at the time of ICSI, embryos transferred, embryos frozen, cumulative embryo score (CES) and implantation and pregnancy rates were compared between the two groups. CES was calculated as the product of blastomere number and embryo grade based on percentage fragmentation (descending scale of 5–1) at the 48 and 72 h period of assessment (Neal et al., 2005a). Higher CES indicates a better morphological appearance of the embryo (Joesbury et al., 1998; Terriou et al., 2001). Area under the curve (AUC) of E₂ response was calculated by the trapezoidal method to assess stimulation response. On the basis of our clinic policy, a maximum of two embryos were transferred to women aged <34 and a maximum of three embryos to women aged ≥34 on day 3 of a cycle. A positive pregnancy test was characterized by an elevated β-hCG 14 days after transfer and confirmed by a doubling in value 3 days later. Clinical pregnancy was confirmed by a positive fetal heartbeat by ultrasound, 6.5 weeks after a positive pregnancy test (β-hCG). The number of fetal sacs with a positive heartbeat divided by the total number of embryos transferred was used to calculate the implantation rate.

**Isolated rat follicle culture**

Animal work was carried out in compliance with the Guidelines of the Canadian Council of Animal Care (CCAC) and in accordance with approval from the Animal Research Ethics Board (AREB) at McMaster University. Ovaries (n = 63) from 22- to 26-day-old, immature female Wistar rats were aseptically removed at necropsy and minced into 0.5 mm cubes using a no.22 scalpel blade and incubated at 37°C in α-minimal essential medium (α-MEM) containing collagenase (type 1A, 4 mg ml⁻¹) and DNase 1 (0.3 mg ml⁻¹). Incubation was terminated with the transfer of ovarian tissue into α-MEM supplemented with 10% fetal bovine serum (FBS). Individual follicles were isolated under a stereomicroscope, rinsed in fresh media and placed individually into single wells of a 96-well plate containing follicle culture media (FCM) (α-MEM supplemented with HEPES (10 mM), FBS (0.1% v/v), rat serum (1% v/v), ITS+ (1% v/v), ascorbic acid (25 µg ml⁻¹), non-essential amino acids (1% v/v), streptomycin–penicillin (1% v/v) and FSH (Gonal F, Serono) (75 ng ml⁻¹). Preliminary studies undertaken in our laboratory during development of the IRFC established optimal follicle growth with FCM supplemented with 75 ng ml⁻¹ of FSH (data not shown). To reduce experimental variation caused by damage during the collection procedure, only round follicles (80–100 µm diameter) with an intact theca cell layer were selected for culture. Rat follicles were selected with calibrated micropipettes, and follicle diameter and circumference of each follicle was measured using an Olympus microscope connected to an IP Spectrum module to calculate the follicle area. The average of the minimum and maximum diameter was used to account for the asymmetrical proportion of the follicles. These measurements were recorded daily to assess follicle growth characteristics and coded samples were evaluated. Follicle growth was classified into one of three categories: (i) no proliferation or atretic; (ii) proliferating and/or expanding cumulus growth; (iii) early antral.

Spent media from the IRFC was collected and stored at −80°C until analysed for E₂ concentration by a third generation commercial radioimmunoassay (RIA) kit (Diagnostic Systems Laboratories, TX, USA). Briefly, spent media samples were pooled (four to six follicles per treatment group) and subjected to the RIA. The detection limit was 15 pg ml⁻¹. There was no inter-assay variation since all samples were tested with the same commercial kit, and the intra-assay variation was calculated to be 2.7%.

**Statistical analysis**

Values are expressed as mean ± the standard error of the mean (SEM) where applicable. Rates were expressed as percentages, and the significance of differences between rates was determined using the classical χ²-test. Data were checked for homogeneity of variance and normal distribution before analysis by one-way ANOVA. Differences among group mean values were determined by Dunnett’s method. Statistical analysis was performed using SigmaStat (SPSS, Chicago IL, USA). For all statistical procedures performed, a P-value <0.05 was considered significant.

**Results**

**Stimulation and IVF outcomes**

Patient age, days of stimulation, day 3 FSH, number of amps used, peak E₂ level, AUC, number of oocytes retrieved, oocyte maturity, fertilization rate, embryo utilization rate, number of embryos transferred, number of embryos available for freezing and embryo quality at 48, 72 h and at transfer were similar between the two groups (Table I). However, implantation and pregnancy rates were significantly lower in the MS group (12.0 and 11.1%, respectively) when compared with their NS (25.0 and 40.0%, respectively) counterparts (Table II). The total number of oocytes retrieved for the MS group was 110 (range 6–17 per patient) compared with 172 (range 4–18 per patient) for the MS group. Characteristics of study subjects were similar for both the NS and MS groups (Table II).

**Quantification of B[a]P in serum and follicular fluid**

Female patients included in this study smoked an average of 11.5 ± 1.5 (range 3–25) cigarettes per day. Eight (75%) of the male partners reported smoking 11.7 ± 3.0 (range 5–25) cigarettes daily. Occupation comparison taken from the patient intake questionnaire for women in both groups did not reveal any exposure risk from the workplace environment. Chemical analysis showed that the concentration of B[a]P (ng ml⁻¹) was higher in the serum of MS (0.40 ± 0.13; range 0–1.40 ng ml⁻¹) exposed women, although not statistically different from the NS (0.22 ± 0.15; range 0–0.68 ng ml⁻¹) group (Figure 1). However, B[a]P concentration was significantly higher (P = 0.03) in the FF of MS (1.32 ± 0.68; range 0–11.56 ng ml⁻¹) compared with the NS (0.03 ± 0.01; range 0–0.18 ng ml⁻¹) patients (Figure 1). Concentration of B[a]P in FF was positively correlated (r = 0.7; P = 0.01) with female cigarette consumption (Figure 2). No correlation was observed between B[a]P levels in FF and the male smoking habit or between serum B[a]P levels and smoking habit.

**Effect of B[a]P on folliculogenesis**

The average size (± SEM) of the selected rat follicles was similar (P = 0.12) between the control 97.8 ± 1.5 µm and
each of the treatment groups 101.0 ± 1.3, 101.4 ± 1.3, 101.3 ± 1.4, 99.0 ± 1.6 and 103.6 ± 1.4 μm exposed to 1.5, 5.0, 15, 45 and 135 ng ml\(^{-1}\) B[a]P, respectively. Increasing concentrations of B[a]P treatment attenuated FSH-stimulated rat follicle growth in a dose-dependant manner over a 5-day culture period (Figure 3). The percentage change in follicle area (±SEM) was 158.3 (49.6), 99.7 (27.0), 75.7 (19.3), 38.9 (9.8), 30.4 (14.8) and 38.1 (19.2) for the control rat follicles and those exposed to 1.5, 5.0, 15, 45 and 135 ng ml\(^{-1}\) B[a]P, respectively (Figure 3). Of importance is the observation that 1.5 ng ml\(^{-1}\) B[a]P, a concentration similar to what we found in the FF of MS women, resulted in a significant decrease in follicle growth in vitro (37% of control). Growth characteristics of the control rat follicles revealed that 44.6% had no signs of proliferation and 46.2% had expanding cumulus cells with 9.2% demonstrating signs of pre-antral formation (Figure 4). Increasing concentrations of B[a]P (1.5, 5.0, 15.0, 45.0 and 135.0 ng ml\(^{-1}\) B[a]P) in the IRFC media corresponded with decreasing percentages of proliferating follicles (Figure 4). None of the follicles treated with B[a]P reached a stage of pre-antral formation compared with controls (Figure 4).

E\(_2\) measured in the spent media was significantly lower (\(P < 0.001\)) in treatment groups compared with control rat follicles beginning at 5.0 ng ml\(^{-1}\) B[a]P group (Figure 5). Although not statistically different, the spent media from rat follicles exposed to 1.5 ng ml\(^{-1}\) B[a]P had less than half (0.97 ± 0.61 ng ml\(^{-1}\)) the level of E\(_2\) compared with levels found in the spent media of controls (2.09 ± 0.54 ng ml\(^{-1}\)) (Figure 5).

**Discussion**

Results of this study confirm and expand the evidence that tobacco smoking has a deleterious effect on reproduction. First, we demonstrated that implantation and pregnancy rates were significantly lower in the group of women who smoked compared with their NS counterparts. Furthermore, we report for the first time that B[a]P residues can be measured in serum and FF and are higher in the FF of female smokers. Using an IRFC system, we show that B[a]P at levels representative of the concentration found in FF of women smokers significantly inhibited follicle growth. In addition, increasing concentrations of B[a]P treatment induced decreased E\(_2\) production of the in vitro cultured rat follicles.

Earlier onset of menopause, longer time to natural conception and reduced success with assisted conception have been well documented in women exposed with smoke compared to non-smokers (Augood et al., 1998; Hull et al., 2000; Zenzes, 2000). In the current study, smoking did not have an effect on ovarian response of women undergoing...
IVF treatment. However, despite similar appearing embryo quality, there was a marked difference in pregnancy rate of MS compared with NS women. Women in both the MS and NS groups were similar in age, reasons for infertility and response to treatment with the exception that more women in the MS group were diagnosed with tubal infertility. The stage at which the reproductive process is derailed by smoking has not yet been determined. However, the detrimental effect of smoking becomes more evident in older women undergoing IVF treatment (Sharara et al., 1994; Hughes and Brennan, 1996; Zenzes et al., 1997). This evidence suggests that accelerated oocyte atresia may be one reason behind the shortened reproductive lifespan of women who smoke. Aside from follicle atresia, compromised oocyte quality is another factor contributing to reproductive success or failure.

Cigarette smoke contains nearly 4000 chemicals (Rustemeier et al., 2002; Lodovici et al., 2004), many of which have been shown to be damaging to the mammalian ovary. Several toxicants derived from tobacco smoke have been detected in FF. FF concentrations of the heavy metal cadmium (Zenzes et al., 1995), a known ovarian toxicant, are higher in smokers than in

**Figure 4.** Representative images of individual follicles (bar = 20 μm) cultured in the presence of 0 ng ml⁻¹ B[a]P on day 0 (a), 3 (b) and 5 (c) of culture compared with individual follicles exposed to 135 ng ml⁻¹ B[a]P on day 0 (d), 3 (e) and 5 (f) of culture and those demonstrating signs of pre-antral formation (g). Increasing concentrations of B[a]P in the IRFC media corresponded with decreasing percentages of proliferating follicles (h). Non-proliferating follicles (black bar); proliferating (white bar); and pre-antral formation (grey bar).

**Figure 5.** Levels of estradiol (ng ml⁻¹) in spent media from the isolated follicle culture assay were inversely correlated with increasing concentration of B[a]P. Results are expressed as mean ± SEM of 7 replicates of 96 follicles per culture. (*P < 0.001).
non-smokers. Likewise, cotinine concentrations measured in the FF aspirates of women at the time of oocyte retrieval are positively correlated with the number of cigarettes consumed (Zenzes et al., 1996). Although prior studies have successfully documented the presence of persistent chlorinated organic contaminants in the serum and FF (Jarrell et al., 1993; Younglai et al., 2002), this is the first report to demonstrate that B[a]P, a toxic chemical with a suspected short half-life (Piskova et al., 2005), can be measured in human serum and FF. Goldman et al. (2001) have demonstrated a dose-dependent relationship between the levels of B[a]P and smoking with a 2-fold higher concentration in lung tissue of smokers compared with non-smokers (Goldman et al., 2001).

Although prior studies have documented the presence of B[a]P-induced DNA adducts in human granulosa cells (Zenzes and Reed, 1998), results of the present study are the first to demonstrate that B[a]P can be quantified in human serum and more importantly in ovarian FF. The fact that the levels of B[a]P were higher in FF compared with serum suggests that B[a]P may preferentially concentrate in the ovarian FF. This could be a consequence of the differences in properties between serum and FF or due to the different rates of metabolism. Zenzes (2000) reported DNA adduct formation in granulosa cells, indicating that the ovary has the ability to metabolize B[a]P to toxic metabolites (Zenzes, 2000). However, the relationship between B[a]P levels in serum and ovarian DNA adduct levels remains to be determined, since they have not been linked to follicle demise. B[a]P-induced ovarian toxicity and selective destruction of pre-antral follicles (primordial and primary follicles) have been demonstrated in animal follicles (Mattison, 1980; Benedict et al., 2003); however, the mechanism(s) by which levels of this toxicant (representative of human exposure) causes pre-antral follicle loss is unknown.

We have focused on benzo-[a]-pyrene, which belongs to the PAH group of chemicals. PAHs are known to induce toxic effects through activation of the Ahr. The Ahr is abundantly expressed in oocytes (Robles et al., 2000) and can be triggered by a number of chemicals including B[a]P. Upon ligand binding, the Ahr translocates to the nucleus and interacts with genes that contain a common nucleotide sequence (known as the Ahr response element) resulting in altered expression of Ahr regulated genes (Matikainen et al., 2001). Oocytes in fetal mouse ovaries undergo apoptosis in an Ahr-dependent fashion following PAH exposure (Matikainen et al., 2001). A similar response to PAH has been shown in primordial oocytes from human ovarian tissue (Matikainen et al., 2001). The ovotoxicity of B[a]P may not be limited to induction of primordial follicle demise. A recent study (Benedict et al., 2003) has shown that the Ahr is required for changes in follicle growth but not follicle atresia of pre-antral and antral follicles. These results are contradictory to prior reports (Matikainen et al., 2001), but are consistent with our finding that B[a]P treatment at levels representative of human exposure inhibits growth of isolated rat follicles in vitro.

Additional toxic effects of B[a]P include formation of DNA adducts. Higher levels of DNA adducts in embryos may contribute to failed implantation after overtly normal fertilization (Zenzes et al., 1997; Zenzes, 2000). Since B[a]P can accumulate in the FF, potential toxic effects on folliculogenesis are a concern. The fact that B[a]P can be measured in the serum and more importantly in the FF of women demonstrates that it may have an indirect influence on follicle growth, which may result in compromised oocyte competence. Moreover, evidence that follicle growth is impaired in vitro may be one mechanism causing reduced fertility. Although an in vitro isolated follicle culture assay does not completely mimic the in vivo environment, it provides an excellent model to study the effects of toxicants on follicle development in a time- and stage-dependent manner. In the present study, inclusion of B[a]P in the culture medium inhibited the growth of isolated follicles in vitro. These results are significant because the effect was detectable at concentrations of B[a]P (1.5 ng ml⁻¹) representative of the levels we measured in human ovarian FF. Compared with controls, cumulus expansion and follicle growth were directly affected by this and higher B[a]P concentrations, indicating a toxic effect of B[a]P. The finding that the level of E2 in the spent media of the IRFC2 declined with increasing concentrations of B[a]P suggests that disrupted E2 production may be responsible for the lack of follicle growth. E2 signals primarily via either the estrogen receptor alpha (ERα) or beta (ERβ) (Piskova et al., 2005). Granulosa cells mainly express ERβ, suggesting that this receptor plays a role in granulosa cell growth and modulation of FSH action (Jannongjit and Hammes, 2006). Accordingly, in our culture system, rat follicles exposed to higher levels of B[a]P had reduced E2 output and cumulus cell proliferation.

It is unlikely that a single mechanism of action can explain all of the toxic effects of B[a]P on fertility. In addition to the effects on follicle growth, it is well known that oocytes and cumulus cells exchange numerous factors and communicate through gap-junction channels (Robles et al., 2000; Atef et al., 2005). Environmental toxicants may exert their deleterious effect by disrupting this flow of communication and nutrients from the granulosa cells to the oocyte. Of note, PAHs also cause changes in cellular gap-junction communication (Baird et al., 2005). Dysregulation of these pathways may result in subtle changes in cytoplasmic maturation. Perturbation of this process may allow for the oocyte to ovulate and fertilize normally as shown in this study and reported previously (Neal et al., 2005b), but does not provide the framework to sustain proper preimplantation embryonic development and implantation leading to a viable pregnancy. Furthermore, since the peri-implantation stage appears to be a critical point of embryonic loss among women exposed to cigarette smoke, there may be other toxicant-induced effects on the uterine epithelium by B[a]P and/or other toxicants working alone or in combination to cause implantation failure.

In summary, B[a]P, a constituent of cigarette smoke, can be quantified in the serum and is higher in the FF of women who smoke. Furthermore, we have demonstrated that B[a]P at concentrations representative of the levels we measured in human FF significantly inhibited the growth of isolated rat follicles in culture and reduced the E2 production of these rat follicles. Taken together, our data suggest that B[a]P exposure is a potentially important ovarian toxicant. Ongoing studies...
designed to define the mechanism(s) of action of this toxicant are currently in progress.

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