

# Polycyclic Aromatic Hydrocarbon-Induced Cytotoxicity in Cultured Rat Sertoli Cells Involves Differential Apoptotic Response

Samir S. Raychoudhury and Dana Kubinski

Department of Biological and Physical Sciences, Benedict College, Columbia, South Carolina, USA

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous and persistent environmental contaminants. Some PAHs are carcinogens and may affect the male reproductive system. Therefore, we exposed cultured rat Sertoli cells to a variety of PAHs to determine possible direct toxic effects on the cells of the seminiferous epithelium. Sertoli cells were chosen because they support germ cell development and maintain spermatogenesis. Sertoli cells were isolated from 19–21-day-old male rats and cultured in medium containing 0.08% dimethylsulfoxide as vehicle or in the presence of a variety of PAHs. In the first set of experiments, cultured Sertoli cells were incubated in the presence of  $10^{-4}$  M,  $10^{-6}$  M,  $10^{-8}$  M,  $10^{-12}$  M, and  $10^{-16}$  M fluoranthene (FL) for 24 hr. After 24 hr, FL at  $10^{-4}$ ,  $10^{-6}$ , and  $10^{-8}$  M killed significant numbers of Sertoli cells as revealed by cell viability determinations. Sertoli cells cultured in the presence of  $10^{-6}$  M and  $10^{-8}$  M FL showed morphologic changes. Cell protein levels were decreased and lactate production in the medium increased in a concentration-dependent manner. In addition, Sertoli cells exposed to  $10^{-6}$  M and  $10^{-8}$  M FL exhibited altered F-actin and  $\alpha$ -tubulin distributions compared with untreated controls. Because FL killed about 62% of cells at  $10^{-4}$  M (100  $\mu$ g/mL) and 48% of cells at  $10^{-6}$  M (1  $\mu$ g/mL), increased lactate production about 3-fold at both concentrations, and decreased cell protein by half at  $10^{-4}$  M (100  $\mu$ g/mL), we decided to use a range of concentrations between 10 and 100  $\mu$ g/mL for the second set of experiments using benz[*a*]anthracene (BaA), benzo[*a*]pyrene (BaP), or benzo[*b*]fluoranthene (BbF). After 24 hr, BaA (100  $\mu$ g/mL), BaP (50 and 100  $\mu$ g/mL), and BbF (100  $\mu$ g/mL) significantly increased lactate level in the medium in a concentration-dependent manner. In a third set of experiments, cells were treated in culture uniformly with only 10  $\mu$ g/mL FL, BaA, BaP, or BbF for 24 hr. The cytotoxic effects exerted by these PAHs tested resulted in different apoptotic responses as characterized by *in situ* fluorescence staining. Microscopic analysis of apoptotic cells demonstrated nuclei of reduced size and labeled 3'-OH DNA ends when Sertoli cells had been incubated for 24 hr with 10  $\mu$ g BaP or BbF, but not with vehicle, media, FL, or BaA. Thus, our results demonstrate that the toxic effects of BaP and BbF on Sertoli cells are exerted through apoptosis, whereas FL and BaA do not elicit the apoptotic response. **Key words:**  $\alpha$ -tubulin, benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, F-actin, fluoranthene, lactate, spermatogenesis. *Environ Health Perspect* 111:33–38 (2003). [Online 8 November 2002] doi:10.1289/ehp.5458 available via <http://dx.doi.org/>

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in the air, being present as volatile, semivolatile, and particulate pollutants. Diesel and gasoline engine exhausts, coal and forest fires, tobacco smoke, and electricity-generating power plants are the major sources of environmental PAHs. Some PAHs are known to be carcinogenic (1). Some members of the high-molecular-weight PAH family (e.g., benzo[*k*]fluoranthene) are also known to be carcinogens (2,3) that bind to aryl hydrocarbon receptors (AhRs) and induce ethoxyresorufin-*O*-deethylase (EROD) activity (4). Other members of the low-molecular-weight PAH family [e.g., fluoranthene (FL)] neither significantly bind the AhR nor induce EROD activity (5). However, FL is reported to be toxic in other test systems (6).

Among all types of PAHs found in Murrells Inlet Estuary, South Carolina, FL was about 35% (highest) and benz[*a*]anthracene (BaA) and benzo[*a*]pyrene (BaP) were between 5% and 10% of total PAHs (7). FL, a significant environmental contaminant, has been shown to induce lung tumors in newborn mice

(8) and to induce the formation of DNA adducts (9). Male mice exposed to BaP (10 mg/kg body weight) impregnated 35% fewer females than did control males (10). The impaired fertility in F<sub>1</sub> mice exposed to BaP *in utero* was associated with marked alterations in gametogenesis and folliculogenesis and a dramatic decrease in the size of the gonads, including the reduction in the size of seminiferous tubules in males (10). The present study addresses the potential effects of the identified PAHs separately on Sertoli cells in culture. Some of these compounds, not yet identified as being carcinogenic, warrant further toxicologic investigation. Because FL is the most abundant PAH in the environment (7), it was extensively studied in our culture system. Other PAHs, such as BaA, BaP, and benzo[*b*]fluoranthene (BbF), were included in the second set of experiments as initial screening of the direct toxicity of these compounds to male reproductive cells in culture. In the third set of experiments, all four PAHs (FL, BaA, BaP, and BbF) were included to test apoptotic responses on cultured Sertoli cells. Because Sertoli cells are

the primary cells that create the structural and physiologic environment for spermatogenic cell development and provide a permissive milieu necessary for spermatogenesis (11–13), we have studied cytotoxicity of the selected PAHs on Sertoli cells.

## Materials and Methods

**Animals.** For each Sertoli cell isolation, 10 male CD rats (19–21-day-old albino pups) accompanied by a foster mother were purchased from Harlan Sprague-Dawley (Indianapolis, IN). After 2 days, all male pups were used for Sertoli cell preparation. All animals were housed in a room with controlled lighting (12 hr light/12 hr dark) and temperature (20–22°C).

**Sertoli cell isolation and culture.** Sertoli cells were isolated by a modification of procedures outlined previously (14), as described elsewhere (15,16). The resultant cell fractions obtained after serial enzymatic digestion and subsequent washing were cultured in Dulbecco's modified Eagle's/Ham's F-12 media at a 1:1 ratio with 4 mmol/L glutamine and 15 mmol/L HEPES (DME/F-12), supplemented with 5% fetal bovine serum and antibiotics (17). Sertoli cells were plated onto 22 × 22 mm glass coverslips in a 35-mm diameter culture dish (Falcon Plastics, Lincoln Park, NJ) at approximately  $1 \times 10^6$  cells per dish. Cells were incubated at 33°C in a humidified incubator in an atmosphere of 95% air and 5% CO<sub>2</sub>. After 24 hr, media from all dishes were replaced with fresh media. Subsequently, culture media were changed every 2 days, and the cultures were maintained for a period of 6 days.

On day 3 of culture, contaminating spermatogenic cells were lysed with a hypotonic solution of 20 mM Tris HCl (pH 7.4) for 5 min as described previously (18). To check for possible Leydig cell and peritubular cell contamination in Sertoli cell preparations, Sertoli cell cultures were occasionally stained histochemically for 3 $\beta$ -hydroxysteroid dehydrogenase (14) and alkaline phosphatase (19), respectively. Sertoli cell cultures were virtually negative for Leydig cell contamination and

Address correspondence to S.S. Raychoudhury, Room 206, Alumni Hall, Department of Biological and Physical Sciences, Benedict College, 1600 Harden Street, Columbia, SC 29204 USA. Telephone: (803) 255-1781. Fax: (803) 253-5068. E-mail: sraychoudhury@crle.com

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consistently contained < 1% peritubular myoid cells. In fact, for the experiments described here, *in vitro* cell cultures showed > 95% staining for the positive Sertoli cell marker oil red O, and fewer than 1% of the cells before PAH treatment exhibited staining for the myoid cell marker enzyme alkaline phosphatase. These data are consistent with those of previous cell isolates as described elsewhere (20) and indicate that results obtained here are best explained by direct effects on Sertoli cells and not by effects on minor cell population contaminants. Sertoli cell viability on day 6 of culture was > 95% as assessed by trypan blue exclusion.

After day 6, Sertoli cells in 35-mm culture dishes were incubated in media containing 0.08% dimethylsulfoxide (DMSO) as vehicle, or in the presence of FL at various concentrations ( $10^{-4}$ ,  $10^{-6}$ ,  $10^{-8}$ ,  $10^{-12}$ ,  $10^{-16}$  M). In a second set of experiments, cells were incubated in the medium containing 10, 50, and 100  $\mu\text{g}/\text{mL}$  BaA, BaP, or BbF. After 24 hr of incubation with these treatments, cells were prepared for cytotoxicity study and protein assay, and the media were saved for lactate determinations. The percentage of cell viability and total cell number were determined by trypan blue exclusion. In a third set of experiments, cells were treated in culture with 10  $\mu\text{g}/\text{mL}$  FL, BaA, BaP, or BbF for 24 hr, and apoptotic responses were tested as characterized by *in situ* fluorescence staining and microscopic analysis. We employed a wide range of FL concentrations for the first set of experiments. We then observed that FL killed the maximum number of cells at  $10^{-6}$  M (1  $\mu\text{g}/\text{mL}$ ) and  $10^{-4}$  M (100  $\mu\text{g}/\text{mL}$ ), increased lactate production about 3-fold at both concentrations, and decreased cell protein by half at  $10^{-4}$  M (100  $\mu\text{g}/\text{mL}$ ). For simplification, we limited the use of BaA, BaP, and BbF to 10–100  $\mu\text{g}/\text{mL}$  for the second set of experiments. For the apoptosis study, we compared FL, BaA, BaP, and BbF using only one uniform concentration (10  $\mu\text{g}/\text{mL}$ ). This

relatively high concentration of the tested PAHs enabled us to compare a small number of samples effectively under controlled experimental conditions.

**Cytotoxicity study.** Cell viability was assessed by using a Live/Dead Eukolight viability/cytotoxicity kit (Molecular Probes, Inc., Eugene, OR). This assay was based on the simultaneous determination of live and dead cells with detection of intracellular esterase activity by calcein-AM and of plasma membrane integrity by ethidium homodimer, respectively. This assay was previously described elsewhere in detail (17). A separate cell preparation was used for each cytotoxicity determination. Data were obtained by scanning five randomly chosen fields from each coverslip, with at least 100 cells being analyzed per field. In each cytotoxicity study, each agent was tested on duplicate samples. The values obtained for the duplicate samples were averaged. The average was used for statistical analysis.

**Lactate assay.** Quantitative, enzymatic determination of lactate in media was performed using a lactate assay kit purchased from Sigma Chemical Co. (St. Louis, MO). This assay was routinely performed in our laboratory as previously described (17).

**Protein assay.** Sertoli cells were removed from the plastic dishes. The dishes were rinsed with phosphate-buffered saline (PBS), which was then pooled with cells and sonicated. The pellet was collected in PBS by centrifugation ( $200 \times g$  for 5 min). Cells were stored at  $-70^\circ\text{C}$  until assayed. Sertoli cell protein was determined by the bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as standard.

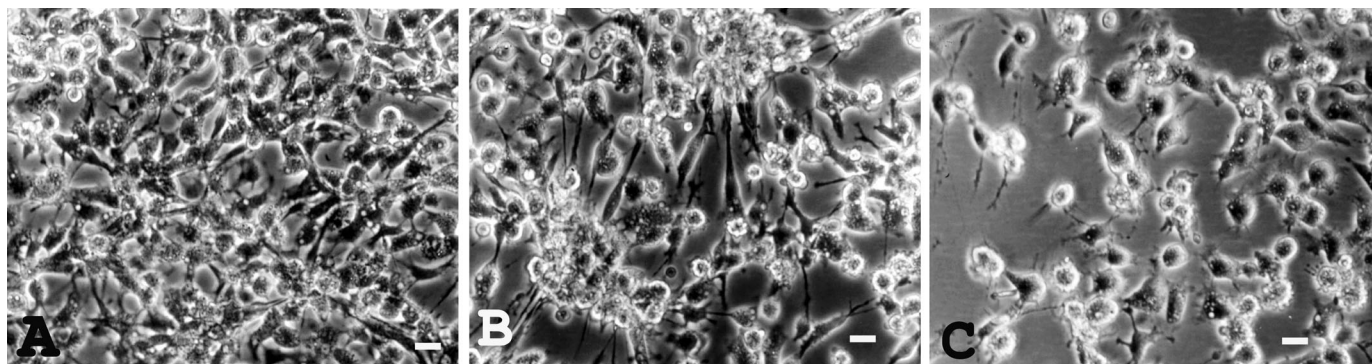
**Confocal microscopy.** F-actin. Coverslips containing fixed Sertoli cells were examined as previously described (17). The coverslips were incubated with 0.25  $\mu\text{g}/\text{mL}$  phalloidin for 30 min. After three rinses with PBS, the coverslips were mounted on microscope slides with Prolong antifade mounting medium

(Molecular Probes). Cells were viewed under a confocal argon-ion laser scanning microscope (MRC-1000; Bio-Rad, Melville, NY).

**$\alpha$ -Tubulin.** The distribution of microtubule protein within cultured Sertoli cells was examined using monoclonal anti- $\alpha$ -tubulin at a final dilution of 1:1,000 (Sigma) and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Alexa-88; Molecular Probes). The coverslips were mounted and examined under a confocal microscope using the same procedures as described above for F-actin staining.

**MTT assay.** The MTT (monotetrazolium) assay, a means of measuring the activity of living cells via mitochondrial dehydrogenases, was performed as described in the MTT assay kit obtained from Sigma. In brief, the media were removed from each culture plate, and the cells were washed with 10 mM sterile PBS. After reconstituting each vial of MTT in 3 mL sterile PBS, 200  $\mu\text{L}$  (10% of the original 2 mL of culture medium) of the MTT solution was added to each cell culture plate. One blank plate was also treated with the MTT solution as described above. Cultured cells were returned to the incubator for 2 hr. After incubation, cultured cells were removed from the incubator. Formazan crystals were dissolved by adding 2 mL (equal to the original culture medium volume) of MTT solubilization solution to each plate, including the blank one. The MTT formazan crystals were completely dissolved after pipetting the solution up and down and gently mixing on a platform shaker. Finally, all samples were read on a Beckman DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA) at a wavelength of 570 nm.

**ApopTag labeling of 3'-OH DNA ends.** We used the ApopTag Plus Fluorescence *In Situ* Apoptosis Detection Kit (Intergen Co., Purchase, NY) following a procedure for cultured Sertoli cells (15) that was modified from the package instructions. The ApopTag labeling method employs the terminal deoxynucleotidyl transferase enzyme to catalyze the



**Figure 1.** Phase-contrast photomicrographs of cultured rat Sertoli cells. Cells were cultured for 6 days in standard medium (DME/F-12), and then maintained for an additional 24 hr in medium containing 0.08% DMSO (A) or FL at  $10^{-6}$  M (B) or  $10^{-8}$  M (C). Sertoli cells treated with these concentrations of FL showed changes in cell shape (B and C) compared with controls (A). The formation of multiple cytoplasmic extensions is evident with FL  $10^{-6}$  M (B), whereas the surrounding cytoplasm appears scant with FL  $10^{-8}$  M treatment (C). Scale bars = 10  $\mu\text{m}$ .



attachment of digoxigenin-labeled nucleotide and unlabeled nucleotide in random sequence onto the free 3'-OH end of apoptotic DNA fragments. Coverslips were counterstained with propidium iodide containing antifade mounting medium. The prepared slides were examined under a Zeiss epifluorescence microscope (Carl Zeiss, Inc., Jena, Germany) using standard filters. These experiments were repeated on three separate Sertoli cell isolations.

**Statistical analysis.** We repeated all experiments at least three times with two or three dishes per group and calculated mean and SEM. Differences among means were compared using one-way repeated-measures analysis of variance (InStat2 software; Graphpad Inc., San Diego, CA) followed by Tukey test with  $p < 0.05$  considered significant.

## Results

Representative phase-contrast photomicrographs of cultured rat Sertoli cells are presented in Figure 1. Sertoli cells incubated for 24 hr in 0.08% DMSO appeared morphologically normal in culture (Figure 1A). Cell-cell contact was common, with large areas of the culture plates showing cellular confluency. In contrast, treatment with  $10^{-6}$  M (Figure 1B) or  $10^{-8}$  M FL (Figure 1C) altered Sertoli cell morphology. Within 24 hr

after plating, Sertoli cells cultured under these conditions were shrunken and retracted, exhibiting cytoplasmic extensions.

Quantitative data from the cytotoxicity experiments are presented in Table 1. The total number of cells did not change with any of the treatments during the culture times assessed. FL directly affected Sertoli cell viability *in vitro* in a concentration-dependent manner. After 24 hr, a significant ( $p < 0.05$ ) reduction in the viability of Sertoli cells was noted with  $10^{-4}$  M,  $10^{-6}$  M, and  $10^{-8}$  M FL. Cytotoxic effects of FL were dose dependent, with molarities of  $10^{-12}$  M or lower exhibiting no significant impairment of Sertoli cell viability.

Data quantifying cell protein contents and lactate production by Sertoli cells treated with FL are presented in Table 2. FL at  $10^{-4}$  M or  $10^{-6}$  M significantly reduced cell protein content ( $p < 0.05$ ). This effect was limited to the higher molarities of toxicant tested. Exposure to FL resulted in a concentration-dependent increase in the amount of lactate production by Sertoli cells in the medium. After 24 hr,  $10^{-4}$  M,  $10^{-6}$  M, or  $10^{-8}$  M FL significantly increased lactate levels ( $p < 0.05$ ). Incubation of Sertoli cells with  $10^{-12}$  M or  $10^{-16}$  M FL for 24 hr did not significantly increase lactate production compared with medium or DMSO vehicle controls.

The distribution of F-actin was examined by confocal laser microscopy using rhodamine-conjugated phalloidin to label filamentous actin. Figure 2 shows photomicrographs of F-actin distribution in cultured rat Sertoli cells after treatment either with vehicle alone (Figure 2A) or with FL (Figure 2B and C). After 24 hr of exposure to  $10^{-6}$  M or  $10^{-8}$  M FL, Sertoli cells exhibited altered actin staining. In controls, almost all cultured Sertoli cells exhibited well-organized stress fibers, as expected for cells *in vitro* (Figure 2A). Cells treated for 24 hr with  $10^{-6}$  M FL (Figure 2B), however, contained no visible stress fibers, but rather exhibited shortened and disorganized actin filaments. Sertoli cells incubated with  $10^{-8}$  M FL also demonstrated disorganized and less intense F-actin staining (Figure 2C).

Figure 3 shows photomicrographs of  $\alpha$ -tubulin distribution in cultured rat Sertoli cells after treatment either with vehicle alone (Figure 3A) or with FL (Figure 3B). After 24 hr of exposure to  $10^{-8}$  M or higher molarities of FL, Sertoli cells exhibited altered and complex microtubule networks that extended into longer processes compared with the untreated control group.

Figure 4 presents results of the MTT assay in Sertoli cells treated with various concentrations of FL. The data indicate a concentration-dependent decrease in mitochondrial activity in these cells with increasing concentrations of FL. With  $10^{-4}$  M,  $10^{-6}$  M, or  $10^{-8}$  M FL, the decrease in the mitochondrial activity was statistically significant.

We also screened the PAHs BaA, BaP, and BbF for possible direct toxic effects on Sertoli cells. Cells were incubated in the medium containing vehicle with 10, 50, or 100  $\mu$ g/mL BaA, BaP, or BbF. After 24 hr of incubation, cells were prepared for the protein assay, and the media were saved for lactate determinations. Table 3 shows lactate production by Sertoli cells exposed to BaA, BaP, or BbF. With higher concentrations of all three PAHs (100  $\mu$ g/mL), lactate production was

**Table 1.** Effects of a single exposure to FL on the viability of cultured rat Sertoli cells.

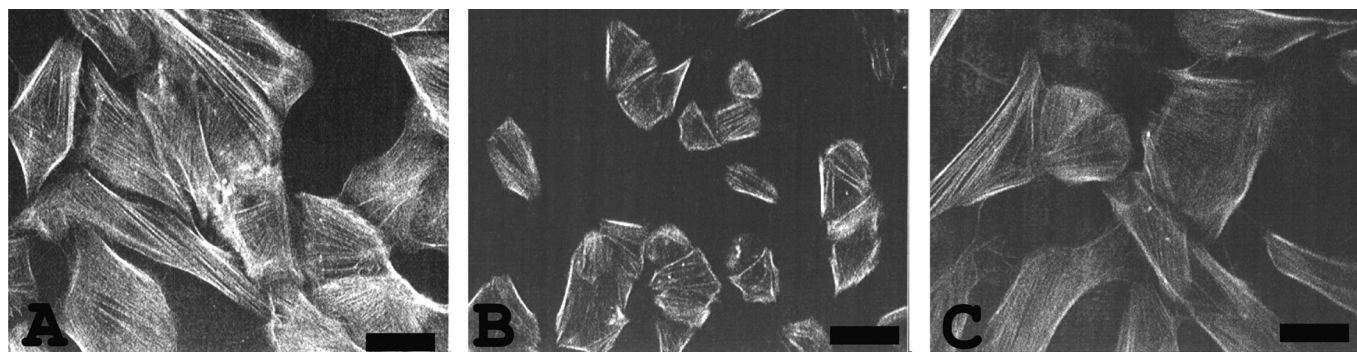
Treatment	Percent viable cells	Total cells ( $\times 10^6$ /mL)
Medium alone	86.3 $\pm$ 0.9 <sup>a</sup>	0.65 $\pm$ 0.04
0.08% DMSO	80.5 $\pm$ 1.5 <sup>a</sup>	0.66 $\pm$ 0.05
$10^{-4}$ M FL	37.5 $\pm$ 0.6 <sup>b</sup>	0.65 $\pm$ 0.03
$10^{-6}$ M FL	51.8 $\pm$ 3.5 <sup>b</sup>	0.64 $\pm$ 0.03
$10^{-8}$ M FL	57.4 $\pm$ 1.9 <sup>b</sup>	0.60 $\pm$ 0.02
$10^{-12}$ M FL	68.8 $\pm$ 2.4 <sup>a</sup>	0.60 $\pm$ 0.02
$10^{-16}$ M FL	87.0 $\pm$ 2.0 <sup>a</sup>	0.62 $\pm$ 0.04

Data represent the mean  $\pm$  SEM from three cytotoxicity studies. The treatments were given at 0 hr, and cell viability was determined 24 hr later. Means in a column with different letters (*a*, *b*) are significantly different from each other ( $p < 0.05$ ).

**Table 2.** Effects of a single exposure to FL on levels of total cellular protein and lactate production by cultured Sertoli cells.

Treatment	Total cell protein (mg/ $10^6$ cells)	Lactate (mg/ $10^6$ cells)
Medium alone	0.41 $\pm$ 0.01 <sup>a</sup>	0.10 $\pm$ 0.008 <sup>a</sup>
0.08% DMSO	0.38 $\pm$ 0.01 <sup>a</sup>	0.11 $\pm$ 0.007 <sup>a</sup>
$10^{-4}$ M FL	0.19 $\pm$ 0.01 <sup>b</sup>	0.32 $\pm$ 0.008 <sup>b</sup>
$10^{-6}$ M FL	0.27 $\pm$ 0.01 <sup>b</sup>	0.30 $\pm$ 0.008 <sup>b</sup>
$10^{-8}$ M FL	0.30 $\pm$ 0.01 <sup>a</sup>	0.20 $\pm$ 0.007 <sup>b</sup>
$10^{-12}$ M FL	0.37 $\pm$ 0.01 <sup>a</sup>	0.14 $\pm$ 0.006 <sup>a</sup>
$10^{-16}$ M FL	0.40 $\pm$ 0.01 <sup>a</sup>	0.12 $\pm$ 0.006 <sup>a</sup>

Data represent the mean  $\pm$  SEM from three cytotoxicity studies. The treatments were given at 0 hr, and lactate and protein were determined 24 hr later. Means in a column with different letters (*a*, *b*) are significantly different from each other ( $p < 0.05$ ).



**Figure 2.** Photomicrographs of rhodamine-phalloidin-labeled filamentous actin distribution in cultured rat Sertoli cells. Sertoli cells were cultured on coverslips for 6 days and incubated for additional 24 hr in medium containing 0.08% DMSO vehicle (A) or in medium with vehicle containing  $10^{-6}$  M FL (B) or  $10^{-8}$  M FL (C). In control medium, almost all cultured Sertoli cells exhibited well-organized actin filament protein (A). Cells treated for 24 hr with  $10^{-6}$  M FL (B) exhibited shortened and disorganized actin filaments. Sertoli cells incubated with  $10^{-8}$  M FL also demonstrated disorganized and less intense F-actin (C). Scale bars = 100  $\mu$ m.

significantly increased. The increase was also noticed with 50 µg/mL BaP.

Results from ApopTag fluorescein *in situ* apoptosis detection in Sertoli cells are illustrated in Figure 5. Sertoli cells exposed to media (Figure 5A), vehicle (Figure 5B), or vehicle containing 10 µg/mL FL (Figure 5C) or BaA (Figure 5D) appeared nonapoptotic as revealed by propidium iodide counterstaining. Sertoli cells exposed to BaA, however, exhibited swollen nuclei and disrupted nuclear membranes (Figure 5D). Fewer cells incubated for 24 hr in 10 µg/mL BaP showed apoptotic conditions and decreasing nuclear size (Figure 5E). Cells exposed to 10 µg/mL BbF exhibited reduced nuclear size and staining for apoptotic DNA fragments (Figure 5F).

## Discussion

The findings presented here represent an examination of the potential direct cytotoxic effects of PAHs on isolated cells of the mammalian seminiferous epithelium. As such, they provide an important complement to whole-animal studies that have previously implicated systemic effects as major mediators of testicular disruption due to possible environmental toxicants. Our data also suggest the importance of examining a variety of individual PAHs for adverse effects on the male reproductive system. Here, we demonstrate that these organic chemicals produce direct cellular toxicity *in vitro*.

This study has potential relevance to the reproductive toxicity study conducted in animals by other researchers using BaP and other PAHs. In pregnant mice, 10 µM BaP was reported to cause decreased embryonic development (21). BaP-induced embryotoxicity was evidenced by initiation of oxidation of DNA, protein, glutathione, and lipids (22). This oxidative stress may correlate well with the present study, which indicates increased lactate production by Sertoli cells *in vitro*.

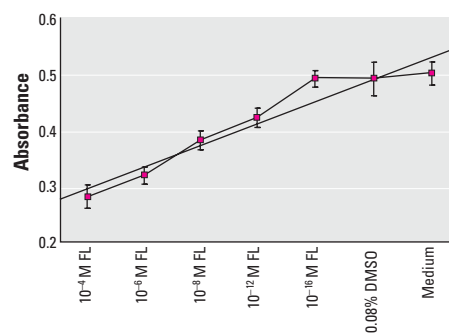
Lactate production by Sertoli cells in the medium was increased by PAHs in a concentration-dependent manner. This indicates that direct cytotoxicity of PAHs impairs Sertoli cell function. Increased lactate levels in medium are usually viewed as evidence of increased cellular glycolysis and/or cell cytotoxicity (17,23). Lactate production by Sertoli cells is important physiologically because developing spermatogenic cells in the adluminal compartment of the seminiferous epithelium have no other source of this compound.

Earlier studies have demonstrated that intraperitoneal injection of BaP into pregnant mice resulted in a greater occurrence of stillbirths, resorptions, and fetal malformations in aryl hydrocarbon (Ah)-responsive mice than in Ah-nonresponsive mice (24). More direct relevance and significance of the present study relied on an even earlier *in vivo* study showing effects of BaP on testicular changes (25); these changes included atrophy of seminiferous tubules, lack of spermatids and spermatozoa, and tumors of the interstitial cells. Research conducted under the Teplice Program in the Czech Republic addressed incidence of cancer and behavioral and reproductive effects (26); human exposure to biomarkers including PAHs resulted in an excess prevalence of low birth weight and premature birth. It was suggested that high levels of air pollution were associated with significant decrements in sperm motility and morphology in samples collected in Teplice during winter months (26) and proportionately more sperm with abnormal chromatin (27).

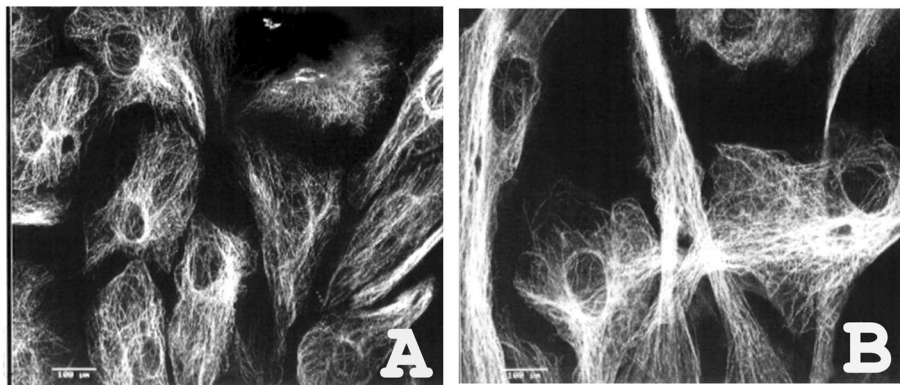
The altered morphology of Sertoli cells treated with FL is reminiscent of the *in vitro* effects of follicle-stimulating hormone (FSH), and dibutyryl cyclic adenosine monophosphate (dbcAMP). The paucity and altered distribution of actin filament stress fibers and microtubular protein within the FL-exposed Sertoli cells are particularly noticeable.

Immature rat Sertoli cells attached to the culture plate and exposed to FSH or dbcAMP manifest long cytoplasmic processes and rounded nuclei (28,29). Similar structural changes were induced by exposing cells to ethyleneglycol-*bis*-tetraacetic acid (EGTA) to chelate Ca<sup>2+</sup> from the cell culture media (29). A spatial association between calcium-binding protein and Sertoli cell microfilaments, and a decrease in the number of microfilaments within Sertoli cells after exposure to FSH, dbcAMP, or EGTA were reported (30). These studies (29,30) suggest that actin filaments are regulated by receptor-mediated cAMP and Ca<sup>2+</sup>-dependent processes and are integral in determining Sertoli cell shape *in vitro*. Other investigators have shown similar effects with 2,5-hexanedione (31). Our findings invite the possibility that FL may exert direct and deleterious effects on such signal transduction events and suggest that experiments be designed to examine such mechanisms.

Sertoli cell microtubules have previously been proposed as a target of 2,5-hexanedione



**Figure 4.** MTT conversion curve showing a concentration-dependent decrease of cell numbers with the increase of FL concentrations (10<sup>-4</sup> M to 10<sup>-16</sup> M). The regression analysis shows linearity of the logarithmic function to the absorbance/cell number curve.  $y = 0.25414 + 3.9500 \times 10^{-2x}$ .  $R^2 = 0.936$ . Data represent mean  $\pm$  SEM from three separate studies.



**Figure 3.** Photomicrographs of fluorescently labeled tubulin distribution in cultured rat Sertoli cells. Sertoli cells were cultured on coverslips for 6 days and incubated for additional 24 hr in medium containing 0.08% DMSO vehicle (A) and in medium with vehicle containing 10<sup>-8</sup> M FL (B). Sertoli cells treated with FL exhibited altered and complex microtubule networks that extended into longer processes (B) compared with controls (A). An intense staining within the bipolar processes of Sertoli cells was observed with FL treatment (B). Scale bars = 100 µm.

**Table 3.** Effects of a single exposure to BaA, BaP, or BbF on lactate production by Sertoli cells.

Treatment	Lactate (mg/mL/mg)
0.08% DMSO	0.25 $\pm$ 0.005
BaA	
10 µg/mL	0.32 $\pm$ 0.004
50 µg/mL	0.35 $\pm$ 0.005
100 µg/mL	0.41 $\pm$ 0.009*
BaP	
10 µg/mL	0.32 $\pm$ 0.001
50 µg/mL	0.39 $\pm$ 0.007*
100 µg/mL	0.49 $\pm$ 0.003*
BbF	
10 µg/mL	0.34 $\pm$ 0.001
50 µg/mL	0.37 $\pm$ 0.007
100 µg/mL	0.41 $\pm$ 0.008*

Data represent the mean  $\pm$  SEM from three cytotoxicity studies. The treatments were given at 0 hr, and lactate was determined after 24 hr of culture.

\*Significantly different from control ( $p < 0.05$ ).



(32,33). The distribution of tubulin within Sertoli cells isolated from 2,5-hexanedione-exposed rats appeared similar to the distribution within Sertoli cells isolated from cryptorchid rats. The immunofluorescent distribution of tubulin also corresponded with the morphologic appearance of cells isolated from 2,5-hexanedione-exposed Sertoli cells. In these cells, the well-defined microtubule network extended into the cytoplasmic processes (31). This morphology is very similar to that seen in Sertoli cells exposed to FL.

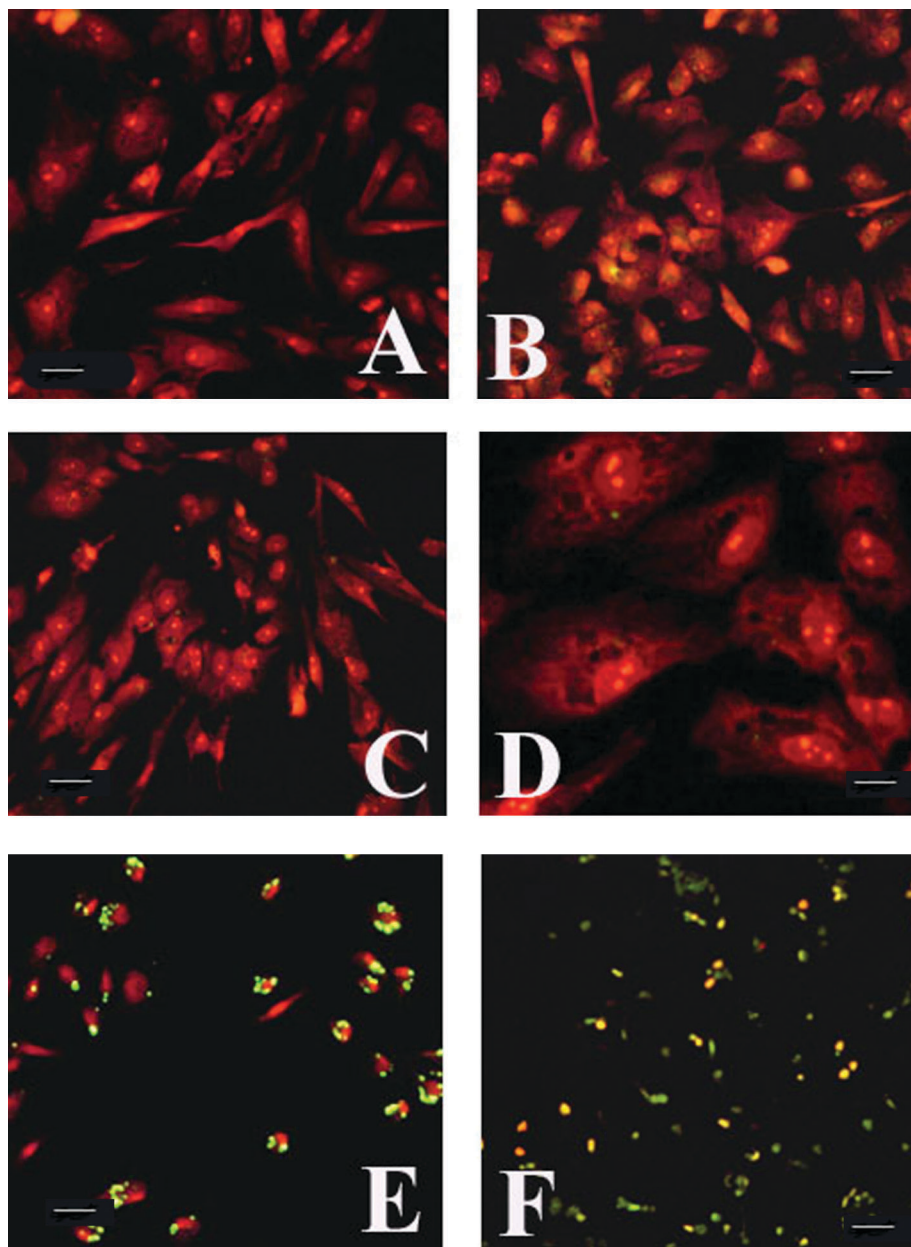
Exposure of Sertoli cells to BaP or BbF, but not to FL or BaA, caused cellular changes that are characteristic of apoptosis (34,35), and obviously BbF elevated the number of nuclei containing 3'-OH DNA ends available for *in situ* apoptosis detection through DNA cleavage. These data strongly suggest that BaP or BbF killed Sertoli cells by apoptosis. FL or BaA did not elicit the same response to induce cytotoxic effects. More recent evidence suggests that disruption of cell signaling pathways involved in the regulation of growth and

differentiation contribute significantly to the toxicity of BaP (36).

The specific PAHs found in highest concentrations in urban air are very similar to the concentrations reported in seawater. In a recent community-based study in Massachusetts, Levy et al. (37) reported that PAH concentrations were greater during the morning rush hour and on weekdays in close proximity to the bus terminal. The PAHs found in that study included FL, BaP, and BaA. Moreover, the long-lived nature of all PAHs and their metabolites, coupled with their bioaccumulation over time, suggests that our current findings be interpreted as possible direct adverse effects upon human and animal reproduction.

## REFERENCES AND NOTES

1. Phillips DH. Fifty years of benzo(a)pyrene. *Nature* 303:468-472 (1993).
2. Mastrangelo G, Fadda E, Marzia V. Polycyclic aromatic hydrocarbons and cancer in man. *Environ Health Perspect* 104:1166-1170 (1996).
3. Shaw GR, Connell DW. Prediction and monitoring of the carcinogenicity of polycyclic aromatic compounds (PACs). *Rev Environ Contam Toxicol* 135:1-62 (1994).
4. Willett KL, Gardinali PR, Sericano JL, Wade TL, Safe SH. Characterization of the H4IIE rat hepatoma cell bioassay for evaluation of environmental samples containing polynuclear aromatic hydrocarbons (PAHs). *Arch Environ Contam Toxicol* 32:442-448 (1997).
5. Willett KL, Randerath K, Zhou GD, Safe SH. Inhibition of CYP1A1-dependent activity by the polynuclear aromatic hydrocarbon (PAH) fluoranthene. *Biochem Pharmacol* 55:831-839 (1998).
6. Boese BL, Lamberson JO, Swartz RC, Ozretich R, Cole F. Photoinduced toxicity of PAHs and alkylated PAHs to a marine infaunal amphiod (*Rhepoxynius abronius*). *Arch Environ Contam Toxicol* 34:235-240 (1997).
7. Vernberg FJ, Vernberg WB, Blood E, Fortner A, Fulton M, Mckellar H, Michener W, Scott G, Siewicki T, Figi KE. Impact of urbanization on high-salinity estuaries in the southeastern United States. *Neth J Sea Res* 30:239-248 (1992).
8. Wang JS, Busby WF Jr. Induction of lung and liver tumors by fluoranthene in a preweanling CD-1 mouse bioassay. *Carcinogenesis* 14:1871-1874 (1993).
9. Wang J-S, Busby WF Jr, Wogan GN. Formation and persistence of DNA adducts in organs of CD-1 mice treated with a tumorigenic dose of fluoranthene. *Carcinogenesis* 16:2609-2616 (1995).
10. Mackenzie KM, Angevine DM. Infertility in mice exposed in utero to benzo(a)pyrene. *Biol Reprod* 24:183-191 (1981).
11. Skinner MK, Norton JN, Mullaney BP, Rosselli M, Whaley PD, Anthony CT. Cell-cell interactions and the regulation of testis function. *Ann NY Acad Sci* 637:354-363 (1991).
12. Jegou B. The Sertoli-germ cell communication network in mammals. *Int Rev Cytol* 147:25-96 (1993).
13. Raychoudhury SS, Millette CF. Multiple fucosyltransferases and their carbohydrate ligands are involved in spermatogenic cell-Sertoli cell adhesion in vitro in rats. *Biol Reprod* 56:1268-1273 (1997).
14. Welsh MJ, Wiebe JP. Sertoli cells: a rapid method for obtaining viable cells. *Endocrinology* 96:618-624 (1975).
15. Raychoudhury SS, Blake CA, Millette CF. Toxic effects of octylphenol on cultured rat spermatogenic cells and Sertoli cells. *Toxicol Appl Pharmacol* 157:192-202 (1999).
16. Newton SC, Millette CF. Sertoli cell plasma membrane polypeptides involved in spermatogenic cell-Sertoli cell adhesion. *J Androl* 13:160-171 (1992).
17. Raychoudhury SS, Flowers AF, Millette CF, Finlay MF. Toxic effects of polychlorinated biphenyls on cultured rat Sertoli cells. *J Androl* 21:964-973 (2000).
18. Galdieri M, Ziparo E, Palombi F, Russo MA, Stefanini M. Pure Sertoli cell cultures: a new model for the study of somatic-germ cell interactions. *J Androl* 5:249-254 (1981).
19. Chapin RE, Phelps JL, Miller BE, Gray TJB. Alkaline



**Figure 5.** ApoptTag labeling of 3'-OH DNA ends to Sertoli cells. These apoptotic cells (green or greenish yellow) demonstrated fluorescein-labeled anti-digoxigenin antibody. Sertoli cell cultures incubated for 24 hr in medium alone (A), in medium containing DMSO as vehicle (B), or in vehicle containing FL (C) or BaA (D) demonstrated mostly propidium iodide counterstain (red) representing nonapoptotic cells. Cells exposed to 10  $\mu\text{g}/\text{mL}$  BaP (E) or BbF (F) demonstrated apoptotic conditions (greenish yellow) as viewed with appropriate filter under a Zeiss fluorescence microscope. Scale bars = 10  $\mu\text{m}$ .

- phosphatase histochemistry discriminates peritubular cells in primary rat testicular cell culture. *J Androl* 8:155–161 (1987).
20. Raychoudhury SS, Blackshaw AW, Irving MG. Hormonal modulation of the interactions of cultured rat testicular Sertoli and peritubular myoid cells. *J Androl* 14:9–16 (1993).
21. Winn LM, Wells PG. Evidence for embryonic prostaglandin H synthase-catalyzed bioactivation and reactive oxygen species-mediated oxidation of cellular macromolecules in phenytoin and benzo[a]pyrene teratogenesis. *Free Radic Biol Med* 22:607–621 (1997).
22. Wells PG, Kim PM, Laposa RR, Nicol CJ, Parman T, Winn LM. Oxidative damage in chemical teratogenesis. *Mutat Res* 396:65–78 (1997).
23. Chapin RE, Gray TJB, Phelps JL, Dutton SL. The effects of mono-(2-ethylhexyl)-phthalate on rat Sertoli cell-enriched primary cultures. *Toxicol Appl Pharmacol* 92:467–469 (1988).
24. Shum S, Jensen NM, Nebert DW. The murine Ah locus: in utero toxicity and teratogenesis associated with genetic differences in benzo[a]pyrene metabolism. *Teratology* 20:365–376 (1979).
25. Payne A. The pathological effects of the intraperitoneal injection of 3,4-benzopyrene in rats and mice. *Br J Cancer* 12:65–74 (1958).
26. Sram RJ, Binkova B, Rossner P, Rubes J, Topinka J, Dejmek J. Adverse reproductive outcomes from exposure to environmental mutagens. *Mutat Res* 428:203–215 (1999).
27. Selevan SG, Borkovec L, Slott VL, Zudova Z, Rubes J, Evenson DP, Perreault SD. Semen quality and reproductive health of young Czech men exposed to seasonal air pollution. *Environ Health Perspect* 108:887–894 (2000).
28. Tung PS, Dorrington JH, Fritz IB. Structural changes induced by follicle stimulating hormone or dibutyryl cyclic AMP on presumptive Sertoli cells in culture. *Proc Natl Acad Sci USA* 72:1838–1842 (1975).
29. Welsh MJ, Van Sickle M, Means AR. Possible involvement of cyclic AMP, calcium, and the cytoskeleton in control of protein secretion by Sertoli cells. In: *Testicular Development, Structure, and Function by Sertoli Cells* (Steinberger A, Steinberger E, eds), New York: Raven Press, 1980:89–98.
30. Means AR, Tash JS, Chafouleas JG, Lagace L, Guerriero V. Regulation of cytoskeleton by  $Ca^{2+}$ -calmodulin and cAMP. *Ann NY Acad Sci* 383:69–84 (1982).
31. Hall ES, Hall SJ, Boekelheide K. Sertoli cells isolated from adult 2,5-hexanedione-exposed rats exhibit atypical morphology and actin distribution. *Toxicol Appl Pharmacol* 117:9–18 (1992).
32. Boekelheide K. 2,5-Hexanedione alters microtubule assembly. II. Enhanced polymerization of crosslinked tubulin. *Toxicol Appl Pharmacol* 88:383–396 (1987).
33. Johnson KJ, Hall ES, Boekelheide K. 2,5-Hexanedione exposure alters the rat Sertoli cell cytoskeleton. I. Microtubule and seminiferous tubule fluid secretion. *Toxicol Appl Pharmacol* 111:432–442 (1991).
34. Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol* 68:251–306 (1980).
35. Schwartzman RA, Cidlowski JA. Apoptosis: the biochemistry and molecular biology of programmed cell death. *Endocr Rev* 14:133–151 (1993).
36. Miller KP, Ramos KS. Impact of cellular metabolism on the biological effects of benzo[a]pyrene and related hydrocarbons. *Drug Metab Rev* 33:1–35 (2001).
37. Levy JL, Houseman EA, Spengler JD, Loh P, Ryan L. Fine particulate matter and polycyclic aromatic hydrocarbon concentration patterns in Roxbury, Massachusetts: a community-based GIS analysis. *Environ Health Perspect* 109:341–347 (2001).