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### Infertility in Mice Exposed in utero to Benzo(a)pyrene<sup>1</sup>

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#### ABSTRACT

The carcinogenic polycyclic hydrocarbon benzo(a)pyrene (BP) is a ubiquitous urban pollutant and is a component of cigarette smoke. Studies reported here were designed to investigate the effect of daily oral doses of 0, 10, 40, and 160 mg BP/kg maternal BW on Days 7-16 of gestation on pregnancy maintenance, and on fetal development and survival of CD-1 mice. Postnatal development and reproductive function also were investigated in F<sub>1</sub> male and female mice exposed to BP in utero.

Total sterility was observed in 97% of F<sub>1</sub> mice exposed prenatally to 40 or 160 mg BP/kg. Fertility was markedly impaired in F<sub>1</sub> animals exposed in utero to 10 mg BP/kg. After 6 months on a breeding study, female mice exposed to this dose of BP in utero gave birth to significantly fewer and smaller litters. Male mice exposed to 10 mg BP/kg impregnated 35% fewer females than did control males. The impaired fertility in F<sub>1</sub> mice exposed to BP in utero was associated with marked alterations in gametogenesis and folliculogenesis and a dramatic decrease in the size of the gonads. Germ aplasia was observed in males as well as a reduction in the size of the seminiferous tubules and an increase in the quantity of interstitial tissue. Microscopic examination of ovarian tissue from F<sub>1</sub> females exposed to 10 mg BP/kg revealed marked hypoplasia with very few corpora lutea or follicles. The data demonstrate a sensitivity of fetal gonads to BP which is similar to effects observed with another polycyclic aromatic hydrocarbon, 9, 10-dimethyl-1,2-benzanthracene (DMBA).

#### INTRODUCTION

The biological properties of polycyclic aromatic hydrocarbons (PAHs) have been studied extensively, and, owing to its potency and wide occurrence, benzo(a)pyrene (BP) has received particular attention. Benzo(a)pyrene and/or its metabolites are potent carcinogens that produce malignant tumors in a variety of tissues, including ovaries, when administered to newborn or mature rodents (Bryan and Shimizu, 1943; Shubik et al., 1960; Herrold and Dunham, 1962; Grant and Roe, 1963; Toth and Shubik, 1967; Jull, 1973; Levin et al., 1977; Kapitulnik et al., 1978; Ketgar et al., 1978). Benzo(a)pyrene is a ubiquitous urban pollutant

(Sawicki, 1967) which is present in the environment in automobile exhaust fumes, in some foodstuffs, and as a by-product of the combustion of bituminous coal-containing fuels. Although BP is present in cigarette smoke condensate with over 300 other PAHs and alkyl-substituted PAHs, BP and/or its metabolites are considered by some to be the most important chemical carcinogens of tobacco smoke (Hoffmann and Wynder, 1972).

Benzo(a)pyrene is a mutagen in in vivo mammalian cytogenetic assays and is capable of inducing significant aberrations of chromosomes in mature mouse oocytes (NMRI-strain) and Chinese hamster bone-marrow cells (Basler and Röhrborn, 1976; Huberman et al., 1976; Ashby and Stiles, 1978). This compound also has the ability to induce somatic mutations in mouse embryos exposed in utero to 2 mg/day on Days 7-10 of gestation (Davidson and Dawson, 1976, 1977).

In addition, mature mouse and rat ovaries (Mattison and Thorgeirsson, 1978); cultured mouse, hamster, and human embryo and fetal cells; and human placenta (Pelkonen, 1976; Juchau et al., 1976; Selkirk, 1977) possess the enzyme system(s) capable of metabolizing

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PAHs, such as BP, to reactive intermediates which have been shown to be toxic, carcinogenic, and/or mutagenic.

The experiments described here were designed to evaluate the effects of prenatal exposure to BP on pregnancy maintenance, fetal development and survival, and postnatal development and reproductive function of F<sub>1</sub> male and female CD-1 mice.

#### MATERIALS AND METHODS

Adult CD-1 female mice that had at least one litter, and proven breeder males (Charles River Breeding Labs., Inc., Wilmington, MA) were acclimated in the laboratory for at least 1 week prior to breeding. Purina lab chow and tap water were provided ad libitum. Pregnant mice were obtained by placing three females with one proven breeder male. The day a vaginal plug was found was considered Day 1 of pregnancy. Mated females were assigned at random to treatment groups and the appropriate dosage of BP (Aldrich Chemical Co., Inc., Milwaukee, WI) in 0.2 ml corn oil was administered daily by oral intubation on Days 7 through 16 of gestation. Control animals received 0.2 ml corn oil. Doses tested were 0, 10, 40, and 160 mg BP/kg maternal BW per day. These doses were selected based on the results of a preliminary study. Each test group consisted of 30 or 60 treated females.

Following treatment, the animals were allowed to deliver at term and nurse their litters through weaning.

Pups were counted at birth (Day 1 of age) and examined for gross abnormalities. At 4 days of age the F<sub>1</sub> young were sexed and weighed, and litter size was reduced to a maximum of eight pups (four males and four females when possible). Litters were allowed to remain with their mothers until postnatal Day 20, at which time the F<sub>1</sub> young were examined and weighed and the dams were discarded. At 7 or 8 weeks of age, young were assigned at random to the Male or Female Breeding Study, respectively. An outline of the procedures followed for each of these studies is given below. Any F<sub>1</sub> animals that were not selected for the breeding studies were sacrificed.

#### Male Breeding Study

Each F<sub>1</sub> male mouse (n = 20–45/group) was housed with two untreated virgin females every 5 days for 25 days (for a total exposure of 10 untreated females/F<sub>1</sub> male). Fourteen days after separation from the males (Days 14–19 of gestation), the females were sacrificed and the number of implants, fetuses, and resorptions was recorded. The F<sub>2</sub> young were examined for gross abnormalities.

#### Female Breeding Study

Each F<sub>1</sub> female mouse (n = 20–55/group) was housed continuously with an untreated proven breeder male for a period of 6 months. If the female failed to produce a litter during the first 30-day period, a new breeder male was introduced. All F<sub>2</sub> young were counted and examined for gross abnormalities on Day 1 of life, and were sexed and weighed at 4 days of age.

TABLE 1. Pup survival following oral administration of benzo(a)pyrene to female mice on Days 7 through 16 of gestation (mean ± SEM).

	Benzo(a)pyrene (mg/kg/day)			
	0	10	40	160
Females treated	60	30	60	30
Females pregnant (%)	46 (77)	22 (73)	46 (77)	15 (50)*
Parturition examination				
Viable litters (%)	46 (77)	21 (70)	44 (73)	13 (43)**
Mean litter size <sup>a</sup>	9.4 ± 0.6	8.6 ± 0.8	10.5 ± 0.5	8.8 ± 0.9
4 Day examination				
Viable litters	45	20	42	12
Mean litter size	9.5 ± 0.6	8.6 ± 0.7	10.5 ± 0.5	8.3 ± 1.0
Mean pup weight (g)	2.7 ± 0.02	2.8 ± 0.04	2.5 ± 0.02	2.2 ± 0.04
20 Day examination				
Viable litters (%)	45 (75)	20 (67)	40 (67)	10 (33)**
Mean litter size <sup>b</sup>	7.1 ± 0.3	7.0 ± 0.5	7.5 ± 0.2	6.5 ± 0.6
Mean pup weight (g)	11.2 ± 0.1	11.6 ± 0.1	10.4 ± 0.1**	9.7 ± 0.2**
42 Day examination				
Viable litters (%)	45 (75)	20 (67)	40 (67)	10 (33)**
Mean litter size	7.0 ± 0.3	6.8 ± 0.5	7.1 ± 0.2	5.9 ± 0.5
Mean pup weight (g)	29.9 ± 0.2	28.2 ± 0.3**	28.0 ± 0.2**	26.8 ± 0.4**

<sup>a</sup>Mean number of live pups per viable litter ± SEM.

<sup>b</sup>All litters were standardized to 8 pups per litter on Day 4.

\*Significantly different from controls (P<0.05).

\*\*Significantly different from controls (P<0.01).

TABLE 2. Reproductive performance of male and female F<sub>1</sub> mice exposed prenatally to benzo(a)pyrene on Days 7 through 16 of gestation (mean ± SEM).

	Benzo(a)pyrene (mg/kg/day)			
	0	10	40	160
Male breeding study				
n F <sub>1</sub> males tested <sup>a</sup>	45	25	45	20
Fertility index <sup>b</sup>	80.4	52.0*	4.7**	0.0**
Mean litter size	11.0 ± 0.1	10.7 ± 0.2	10.8 ± 0.6	...
Female breeding study				
n F <sub>1</sub> females tested <sup>c</sup>	35	35	55	20
Fertility index	100.0	65.7**	0.0**	0.0**
Mean litter size	12.9 ± 0.2	10.4 ± 0.4**	...	...

<sup>a</sup>Beginning at 7 weeks of age, each F<sub>1</sub> male was exposed to 10 untreated females over a period of 25 days.

<sup>b</sup>Fertility index: Females pregnant/females exposed to males × 100.

<sup>c</sup>Beginning at 6 weeks of age, each F<sub>1</sub> female was cohabitated continuously with an untreated male for a period of 6 months.

\*Significantly different from controls (P<0.05).

\*\*Significantly different from controls (P<0.01).

On Day 20 postpartum the young were examined, weighed, and sacrificed, and the F<sub>1</sub> female was left with a proven breeder male until the conclusion of the study.

#### Histologic Study

To determine what effect BP may have on gonadal morphology, groups of 10 F<sub>1</sub> male and female mice exposed in utero to 0, 10, or 40 mg BP/kg were subjected to a gross necropsy at 6 weeks of age. Reproductive tissues were removed, trimmed, weighed (testes only), fixed in alcohol-formalin-acetic acid

(AFA), sectioned, stained with Harris's hematoxylin and eosin, and prepared for microscopic examination. The gonads of each animal were sectioned transversely through the longest diameter of the organ. Additional serial sections were deemed unnecessary since the observed lesions were approximately equal in number and were uniformly distributed throughout the section.

#### Statistical Evaluation

Data on pregnancy maintenance and fetal survival



FIG. 1. Total number of litters born to F<sub>1</sub> female mice exposed to 10 mg BP/kg in utero on Days 7 through 16 of gestation and to control mice.

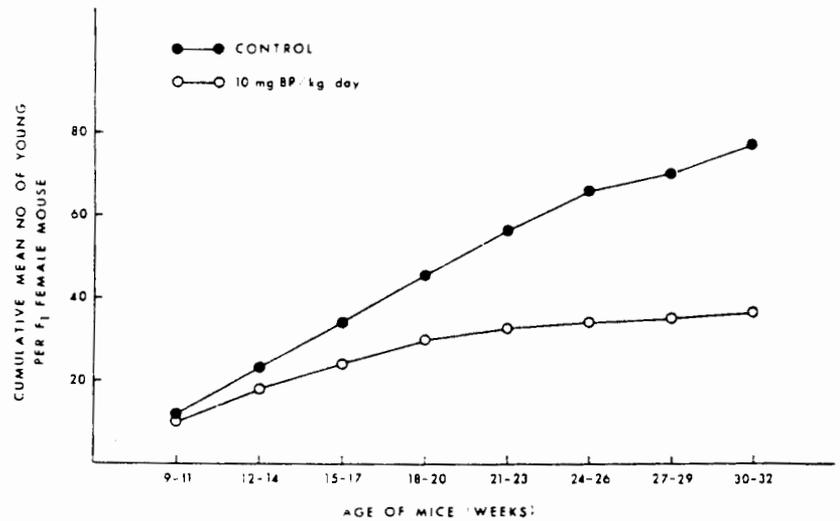


FIG. 2. Cumulative mean number of young born to F<sub>1</sub> female mice exposed to 10 mg BP/kg in utero on Days 7 through 16 of gestation and to control mice during successive 3 week intervals.

were analyzed for statistical significance by appropriate nonparametric procedures (Siegel, 1956). The least significant difference method was used to analyze data on litter size, and fetal and testes weights (Steel and Torrie, 1960).

#### RESULTS

Benzo(a)pyrene was not toxic to mothers or fetuses at any of the doses tested (Table 1). Pregnancy maintenance was reduced ~25% at the 160 mg dose level; however, all litters produced by females treated with BP appeared normal by gross observation. Although there were no statistically significant differences in litter size among treated and control groups, there was a significant decrease at 20 and 42 days of age in weights of pups from treated dams.

In the male breeding study, all of the males in the control group were fertile (i.e., sired at least one litter), and they impregnated 80% (362 of 450) of the females to which they were exposed (Table 2). Twenty of the 25 males exposed to 10 mg BP in utero were fertile and

impregnated only slightly more than one-half (130 or 250) of the females to which they were exposed. Only 3 of 45 males exposed to 40 mg BP were fertile and males exposed to 160 mg BP did not impregnate any females.

After 6 months on the breeding study, 11 litters were produced by F<sub>1</sub> females exposed in utero to 40 to 160 mg BP, and 34% of the females from the treatment group exposed to 10 mg BP were infertile (Table 2). In addition, animals in the 10 mg treatment group that became pregnant were subfertile and gave birth to significantly smaller litters and fewer litters (Table 2, Fig. 1).

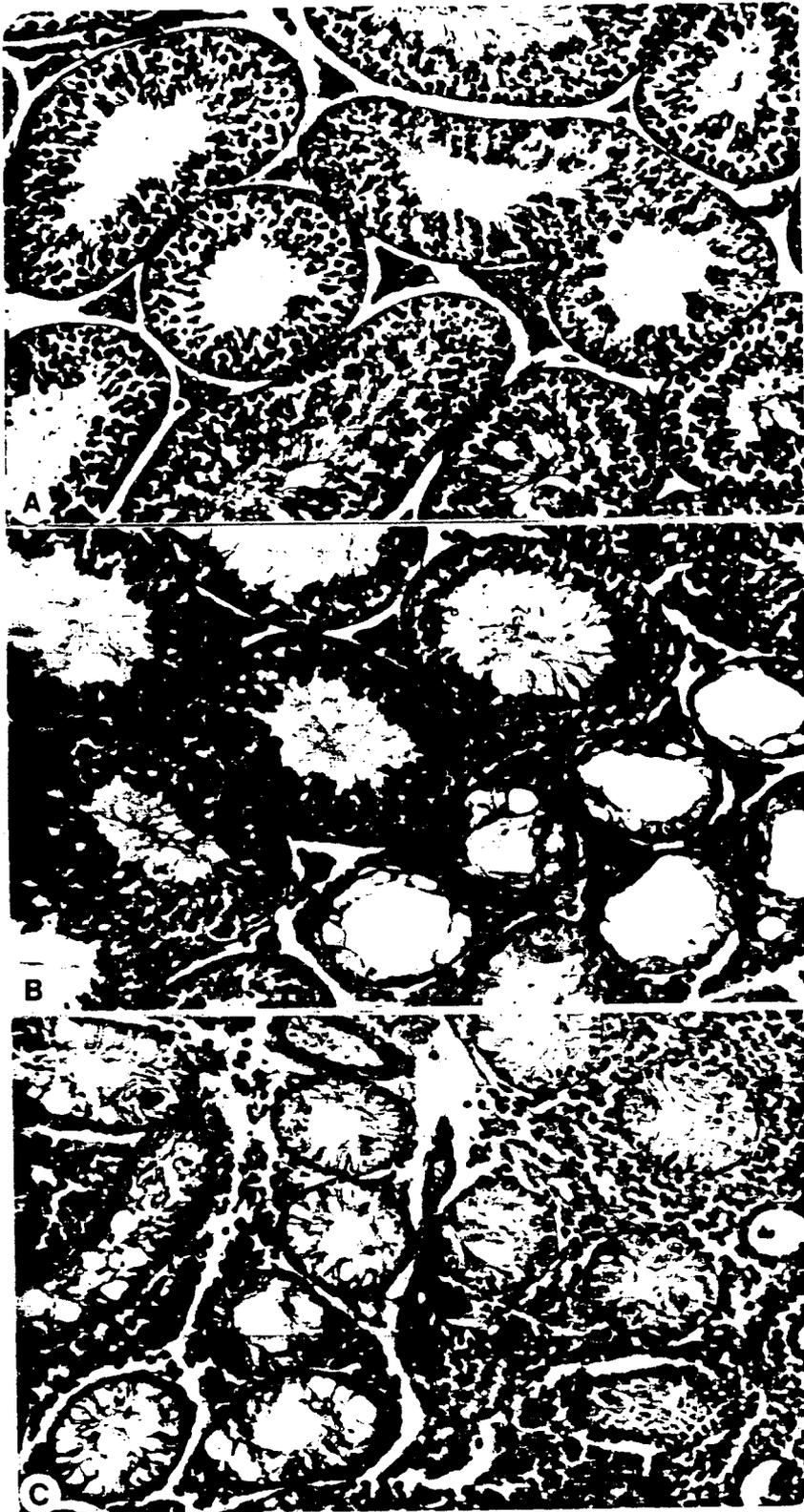
Reproductive capacity may be defined as the total number of young a female produces during forced breeding, and is used as an indication of reproductive competence (Peto and Levy, 1964). Mice in the control group had a mean reproductive capacity of 78 young when they were 32 weeks old, and more young would have been born after this age as suggested by the curve (Fig. 2). However, mice

FIG. 3. Cross sections of testes.  $\times 11.3$ .

A) Control mouse. Seminiferous tubules are of normal size with active spermatogenesis. Interstitial cells and Leydig cells are evident in normal numbers.

B) Mouse exposed in utero to 10 mg BP/kg. Several normal tubules with active spermatogenesis are adjacent to seven atrophic small tubules. The small tubules consist of prominent basement membrane surrounding vacuoles that have replaced degenerated cells. The number of interstitial cells between these tubules appears to be increased.

C) Mouse exposed in utero to 40 mg BP/kg. Tubules are severely atrophied and essentially aspermic. Interstitial cells are quite prominent.



posed to 10 mg BP in utero achieved a maximum reproductive capacity of only 30 to 35 young by 20 weeks of age. This represents a 60% reduction in reproductive capacity.

There were no gross abnormalities in  $F_2$  offspring from the  $F_1$  reproduction studies, and there were no significant differences among treatment groups in  $F_2$  body weights at 4 or 20 days of age in the female breeding study (data not presented).

The most obvious effect of prenatal exposure to BP was a dramatic decrease in the size of the gonads. Paired testes weights were significantly reduced, and testes from animals exposed in utero to 10 mg BP weighed ~60% of the weight of testes from control animals (132 vs 228 mg); testes from animals exposed to 40 mg BP weighed ~18% of controls (41 vs 228 mg).

Histologic examination of gonadal tissue sections revealed significant alterations following exposure to BP. Atrophic seminiferous tubules of males exposed to 40 mg BP (Fig. 3C) were smaller than tubules from control animals (Fig. 3A) and were empty except for a basal layer of cells. There also was a pronounced increase in the number of interstitial cells in the testes of males exposed to BP. It is uncertain whether this apparent increase is real or the result of the marked decrease in tubule size.

Testicular damage with the lowest dose of BP tested (10 mg) appeared to be partial (Fig. 3B). Although the testes of all males in this treatment group showed evidence of tubular injury, each animal also had some seminiferous tubules that displayed active spermatogenesis.

The gonads of  $F_1$  females exposed to BP in utero also showed a marked treatment effect. Since most of these animals either had no ovaries or only remnants of ovarian tissue, ovarian weights were not recorded. Microscopic examination of tissues available from  $F_1$  females exposed to 10 mg BP/kg revealed that they were hypoplastic with very few follicles and corpora lutea (CL) (Fig. 4B). This is in contrast to control animals where all stages of follicular development, including CL, were

present (Fig. 4A).

Ovarian tissue fragments from females in the 40 mg treatment group also were hypoplastic and showed no evidence of folliculogenesis; they consisted of lutein cells, medullary cords, and very little interstitial tissue (Fig. 4C).

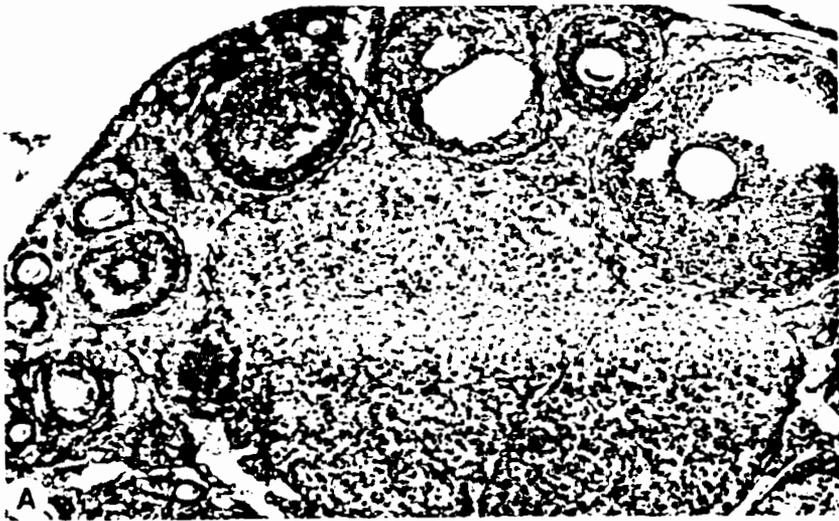
#### DISCUSSION

Sterility was observed in male and female mice exposed in utero to 40 or 160 mg BP/kg maternal BW per day on Days 7 through 16 of gestation (Table 2). Fertility was markedly reduced in animals exposed to 10 mg BP (Table 2, Figs. 1, 2). This infertility was associated with dramatic alterations in gonadal morphology and germ cell development (including impaired gametogenesis and folliculogenesis, which were apparent in both sexes by 6 weeks of age after prenatal exposure to 40 mg BP (Figs. 3C, 4C). The subfertility observed in  $F_2$  males from the 10 mg treatment group may be explained by the impaired testicular function (based on morphological criteria) observed at 6 weeks of age (Fig. 3B). Similarly, the depletion of oocytes and reduced folliculogenesis observed in  $F_1$  females exposed to 10 mg BP (Fig. 4B) probably accounts for the impaired fertility in this group of animals.

The rat placenta is highly permeable to BP administered to the dam on Day 21 of pregnancy in a single oral dose of 200 mg/kg (Alexandrov, 1976). The accumulation of BP in rat fetuses was maximal 3 h after administration of the compound, and only trace amounts of BP were detected in fetal tissues 5 h after treatment. It seems likely, therefore, that the effects of BP on the reproductive capacity of the  $F_1$  generation are related to prenatal exposure to the compound rather than being due to postnatal exposure via the dam's milk or excreta.

To the best of our knowledge, this is the first time anyone has reported an effect of BP on male reproductive function. However ovarian sensitivity to BP has been reported previously by Mattison and Thorgiersson

FIG. 4. Cross sections of ovaries.  $\times 7.1$ .  
 A) Control mouse. Folliculogenesis is normal and CL are present.  
 B) A mouse exposed in utero to 10 mg BP/kg. Folliculogenesis is virtually absent, and the section consists largely of luteal cells.  
 C) Mouse exposed in utero to 40 mg BP/kg. Folliculogenesis is absent, and tissue consists largely of stromal elements interspersed with luteal cells.



(1978) who observed that a single i.p. injection of 20 mg BP/kg destroyed 25% of the primordial oocytes in ovaries of weanling mice; 98% of the oocytes were destroyed at a dose of 240 mg BP/kg.

Both cigarette smoke and polluted urban air contain very small amounts of BP. Wynder and Hoffman (1972) reported values ranging from 18 to 47 ng of BP in the smoke from a single cigarette or cigar smoked mechanically. The concentration of BP in the atmosphere of several large cities in the United States varies from a low of  $0.3 \mu\text{g}/\text{m}^3$  in summer to a high of  $7.4 \mu\text{g}/\text{m}^3$  in winter (Sawicki, 1967). These amounts would be roughly equivalent to an exposure rate of  $\sim 0.05$  to  $25 \mu\text{g}/\text{kg}$  BW per day. These levels are 1/200,000 to 1/400 the minimum daily effective dose in this study (10 mg/kg). However, if one considers a comparable period of exposure in a pregnant human female ( $\sim 50\%$  of the gestation period or 140 days) the total dosage ranges from  $\sim 0.005$  to  $3.5 \text{ mg BP}/\text{kg}$  or 1/9000 to 1/25 the total dose of 90 mg/kg used in this study. The reader must bear in mind that these comparisons are based on an oral vs an inhalation route of exposure and are not based on levels of circulating BP.

The results of studies with BP reported here and previous data from our laboratory concerning effects of DMBA on reproductive function (MacKenzie et al., 1979) demonstrate that the developing fetal mouse gonad is adversely affected by prenatal exposure to PAHs which are present in cigarette smoke and are common urban pollutants. Whether these observations have any clinical significance remains to be seen. However, if human adult or fetal gonads respond in a similar manner, chronic exposure of adults to these compounds could increase the risk of infertility in these individuals and/or in their offspring.

This risk may be particularly great in persons who smoke cigarettes. Several groups of investigators have suggested that an apparent correlation exists between smoking and the premature onset of spontaneous menopause (Jick et al., 1977; Baily et al., 1977). This effect has been attributed to premature depletion of germ cells under the influence of PAHs (Mattison and Thorgeirsson, 1978).

The mechanism(s) by which PAHs and/or their reactive intermediates interfere with gonadal development and reproductive function is not known. Studies are in progress to establish the optimum period of exposure required

for PAH-induced reproductive dysfunction, to determine what factors (endocrine and otherwise) are responsible for the gonadal dysfunction and to establish whether related chemicals exert effects similar to BP and DMBA. In addition the potential tumorigenic effects of PAHs in the offspring of treated pregnant mice are also being investigated.

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