Acute and Subchronic Oral Toxicities of Benzo[a]pyrene in F-344 Rats

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We have studied the acute and subchronic oral toxicities of benzo[a]pyrene (BaP) in male and female F-344 rats. Single acute BaP doses of 0, 100, 600, and 1000 mg/kg dissolved in peanut oil were administered by oral gavage. Subchronic doses of 0, 5, 50, and 100 mg/kg/day were administered for 90 days in the animal diet. The major toxicological endpoints examined included animal body weight, selected tissue weights, and histopathological examinations (liver, kidney, stomach, prostate, testes, and ovaries). In addition, we examined blood elements: red blood cells (RBC), white blood cells (WBC), hemoglobin (Hgb), hematocrit (Hct), mean cell volume (MCV), mean cell hemocrit (MCH), and mean cell hemoglobin concentration (MCHC), blood chemistry (ALT, AST, and BUN), and urine chemistry (glucose, bilirubin, specific gravity, pH, protein, urobilinogen, nitrite, occult blood, and leukocytes). In the acute study, WBC were significantly decreased and mean cell-hemoglobin concentration was significantly increased, both in males only. The liver:body weight ratio was significantly increased in males and females (up to 30%). None of the blood chemistry or urine parameters were significantly affected. In the subchronic study, mean body weight was significantly decreased in males only (13%), and the liver:body weight ratio in males was significantly increased. Several of the blood elements were significantly decreased in males and females after 90 days; RBCs (up to 10%), Hct (up to 12%), and Hgb (up to 12%). For blood chemistry parameters (AST, ALT, BUN), only BUN in males was significantly increased in the high dose group (100 mg/kg) at the 90 day time point. The histopathological examination of selected tissues showed significant abnormalities (tubular casts) only in the male kidney, at the 2 highest doses, after 90 days. These studies indicate that the acute and subchronic toxicities of BaP are relatively low, BaP affects specific blood elements and organs, and BaP has a greater effect on males than females. The induction of non-carcinogenic kidney abnormalities in males only may be indicative of renal dysfunction and further substantiates an apparent sex difference in tolerance to BaP.

Key Words: benzo[a]pyrene; acute; subchronic; toxicity.

Benzo[a]pyrene (BaP) is a member of the polycyclic aromatic hydrocarbon (PAH) family, which includes more than 100 different compounds. These compounds are formed from natural and man-made sources, but BaP originating from man-made sources are quantitatively the most significant (ATSDR, 1995). Because BaP is formed during high temperature pyrolytic processes, the compound is ubiquitous in the environment and virtually all direct releases of BaP into the environment are to the atmosphere. Historically, the greatest releases to the residential environment have been attributed to home heating using coal or wood (EPA, 1985). Other sources of BaP exposure may occur as a result of cigarette smoking (Adams et al., 1987), consumption of contaminated foods (Phillips, 1999) or water (EPA, 1985), and occupational exposures (Arnould et al., 1999; Boffetta et al., 1997; Mumford et al., 1993; Pastorelli et al., 1996). BaP has also been found at a large number of National Priorities List (NPL) or Superfund hazardous waste sites (ATSDR, 1995). A total human environmental exposure study (Waldman et al., 1991) revealed that inhalation of contaminated air and ingestion of contaminated food are the major routes of exposure to BaP.

The non-carcinogenic toxicity of BaP has been studied in animals by a number of investigators (De Jong et al., 1999; Legraverend et al., 1983; Silkworth et al., 1995) and the major effects attributable to BaP appeared to occur in the liver, the hematopoietic and reproductive systems, and the kidney. However, most of the in vivo toxicity studies involving BaP were conducted via dermal, intravenous, or inhalation exposures. Very few studies have been performed through the oral route (reviewed in ATSDR, 1995). With the exception of De Jong et al. (1999), the existing oral studies were limited to a single-dose acute exposure. Also, no attempt had been made to study the BaP toxicity in a subchronic regimen. Therefore, the quantitative risk assessment of BaP has been hampered by the lack of sufficient data sets from animal studies. Also, no human studies documenting the specific non-carcinogenic toxic effects of BaP have been found in the literature (ATSDR, 1995). For a complete risk estimation, long-term animal exposure to BaP is essential (Collins et al., 1991). Also, information relative to acute and subchronic exposures through ingestion of BaP-contaminated water, food, or soil by humans is vital in assessing the risk to individuals living in areas surrounding hazardous waste sites.

Due to the widespread availability of BaP in the environment and the great potential for human exposure, the potential...
adverse effects of this compound merits investigation. Hence, the objective of this study was to determine the dose-related oral acute and subchronic toxicities of BaP on a number of organs, tissues, blood parameters, and blood chemistry, using rats as the animal model.

MATERIALS AND METHODS

Animals

Animal care was in conformity with the NIH guidelines, Care and Use of Laboratory Animals (NIH-78–23). Male and female Fisher (F-344) rats (Harlan Laboratory, Indianapolis, IN) weighing approximately 130–160 g (~8 weeks of age) were used throughout this study. The animals were housed by sex, in groups of 3–4 per cage, maintained on a 12/12-h. light/dark cycle (lights on at 0600 h) and allowed free access to food and water. All animals were allowed a 7-day acclimation period prior to acute or subchronic dosing, randomly assigned to a control or treatment group and fasted 24 h prior to BaP dosing. After treatment, animals were sacrificed by decapitation under carbon dioxide anesthesia and various endpoints were determined.

Procedure

**Acute studies.** An equal number of male and female (n = 10) animals were assigned to a control group or 1 of 3 treatment groups (100 mg/kg BaP, 600 mg/kg BaP or 1000 mg/kg BaP). As BaP is a potential carcinogen, it was handled in accordance with NIH guidelines (NIH, 1981). The test chemical was dissolved in peanut oil and administered by gavage in a single dose in a volume not to exceed 10-ml/kg body weight. The high dose of 1000 mg/kg is representative of the maximum concentration of BaP that would completely dissolve into the vehicle. The control animals received the vehicle (peanut oil) only. Fourteen days after dosing, the animals were sacrificed and trunk blood was allowed to clot for subsequent analysis of blood-element levels: red blood cells (RBC), white blood cells (WBC), hemoglobin (Hgb), hematocrit (Hct), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), and blood chemistry levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and blood urea nitrogen (BUN). Blood-element levels were determined using a Coulter Counter Model S770 (Coulter Electronics, Inc., Tampa, FL), and blood chemistry was determined using Sigma Diagnostic Kits (Sigma Chemical Co., St. Louis, MO). Selected tissues and organs (stomach, liver, kidney, testes, and ovaries) were removed to obtain weights and for gross and histopathological examinations. Histology sections of selected tissues were fixed in 10% formalin and stained with hematoxylin and eosin. The sectioning and staining were performed by Oral Pathology Diagnostic Services, Meharry Medical College, Nashville, TN.

**Subchronic studies.** An equal number of male and female (n = 40) animals were assigned to a control group or 1 of 3 nominal treatment groups (5, 50, or 100 mg/kg BaP). The high dosage was selected based upon a preliminary range-finding study showing animals refusing to consistently consume the diet mixture when concentrations were greater than 200 mg/kg. The test chemical (98% pure BaP-Sigma Chemical Co.) was blended into the animal diet (5001 Lab Meal, Purina Ralston Co., St. Louis, MO) in sufficient quantities to achieve the nominal dosages. The animals were allowed the diet mix and water ad libitum. The stability of BaP in the diet was determined to be at least 7 days. Concentrations of BaP in the diet were adjusted every 3–4 days to achieve the targeted dose levels. Animal weights were recorded twice a week. The amount of the diet consumed was estimated by measuring the amount of food retained in the feeding jar at the end of each feeding period and the amount spilled, and subtracting those values from the amount of food given at the beginning of the feeding period. Over the entire exposure period, the total food intake for each treatment group per week was calculated by summation of the measured food intake from each feeding period in that week. These values were then compared against the food-intake data of control rats and represented as percentage gain or loss in dietary intake relative to the controls. The actual intake of BaP by the rats was less than or equal to 10% of the calculated intake. Since a variation of this magnitude is considered acceptable for studies in which the chemical is administered through diet, our nominal doses were not corrected for actual doses.

Urinalyses were performed using the Multi-stix reagent test strips (Bayer Corp. Elkhart, IN) and results were read using a Clinitek 100 urine chemistry analyzer (Bayer). Parameters measured included glucose, bilirubin, ketone, specific gravity, blood, pH, protein, nitrite, urobilinogen, and leukocytes. Animals were sacrificed at 30-, 60-, and 90-day time points to determine specific endpoints as detailed above.

**Statistics**

Data were analyzed using a variety of techniques including the 2-way analysis of variance (ANOVA) for the blood and organ weight data. Analysis of the histopathological data was accomplished using Fisher’s Exact Test and the Cochran-Armitage test for linear trends. An alpha level of >0.05 was used to indicate statistical significance.

RESULTS

**Acute Data**

Of the parameters measured during the acute investigations, only an increase in liver weight and changes in 2 specific blood parameters proved to be significantly different from control animals. Table 1 shows that the liver weight (as determined via
the liver:body-weight ratio) in males and females is significantly increased (up to 30%) from controls and in a dose-dependent manner. None of the other tissues examined showed any significant weight changes. Table 1 also shows a significant decrease in white blood cell (WBC) counts among male animals acutely treated with BaP at 600 and 1000 mg/kg. While control female animals had lower WBC counts than male animals, no significant depression in WBC counts among females was observed. A small but statistically significant increase in mean cell hemoglobin concentration (MCHC) was observed among the male treatment groups. No significant increase was observed among the female treatment groups. None of the other blood elements or the blood chemistry parameters measured was significantly different from the control groups.

Subchronic Data

Food consumption among males was significantly decreased by (10%) in the 100-mg/kg BaP (high-dose) group over 90 days, but female food consumption was not significantly decreased. Similarly, significantly lower body weights (13%) were observed among males in the high-dose groups at both 60 and 90 days. Female body weights in the high-dose groups were decreased only 4% and 6%, respectively, over 60 and 90 days. Liver:body weight ratios were significantly increased in male animals (23%) after 90 days, but there was no significant change in female liver weights. None of the other tissue weights examined was significantly different from the control groups.

Of the blood parameters tested, only the red cell-associated parameters appeared to be affected by subchronic dosing with BaP. Figure 1 shows that RBC counts are significantly decreased in males at dosages of 50 and 100 mg/kg/day at the 60- and 90-day time points. Figure 2 shows that RBC is significantly depressed in the high-dose group only after 90 days. Figures 3 and 4 show that hematocrit levels, in both males and females, are significantly decreased. Males treated at dosage levels of 50 and 100 mg/kg BaP, after 60 and 90 days, showed a decrease up to 12%. The hematocrit level among females (Fig. 4) was significantly depressed only at the 100-mg/kg dose and only at the 90-day time period. Figures 5 and 6 show that hemoglobin levels are significantly depressed after 90 days, for both males and females, at the 100-mg/kg/day dose level. Hemoglobin also appears to be significantly depressed among females at the 50- and 100-mg/kg/day dose levels at 60 days.

Figure 7 shows the percentage of abnormal kidney tissues occurring in male and female animals treated with BaP at dosages up to 100 mg/kg/day after 90 days. Tubular casts were the most common abnormality found in the kidney and were present in 80 and 100% of the male animals at dosages of 50- and 100-mg BaP/kg/day, respectively. Only 10% of the female animals at the 2 high-dose levels showed significant kidney tubular changes.

DISCUSSION

The doses of BaP used in our studies are higher than the levels found in the general environment. However, they may be...
relevant in cases where the specific populations are chronically exposed to BaP emanating from hazardous waste sites, where chronic intake occurs via food or tobacco, or during occupational exposures. Lioy et al. (1991) estimated that BaP intake ranged from 20 – 800 ng/day in people living in the vicinity of hazardous waste sites contaminated by PAHs. BaP levels in mainstream tobacco smoke is reported to be 20 – 40 ng/cigarette (Kuller et al., 1986) and coke oven workers have been exposed to as much as 42,000 ng/m\(^3\) BaP (Lewtas et al., 1997).

The effects of BaP exposure in this study varied by sex but generally decreased body weight, increased liver weight, decreased levels of certain blood elements, and induced abnormal kidney tissues. Increases in the liver:body weight ratio due to BaP exposure was observed in this study and were previously reported in a PAH study conducted by Danz et al. (1991). At this time, the exact mechanism for the increased liver weight is unknown and the toxicological significance of the liver hypertrophy has not been fully elucidated. Acute oral doses of BaP significantly decreased WBC counts up to 13% below control levels in a dose-dependent manner in male F-344 rats. Chronic suppression of lymphocytes may reduce the response to inflammation and infections and our findings of reduced WBC counts are consistent with reports of immunosuppression associated with BaP exposure (Blanton et al., 1988; De Jong et al., 1999; Krieger et al., 1995). Silkworth et al. (1995) reported

![FIG. 3. Hematocrit levels in F-344 male rats exposed to subchronic oral doses of benzo[a]pyrene. Values are mean ± SD; n = 6–8 animals/dose group; *p < 0.05 (significantly different from controls).](image)

![FIG. 4. Hematocrit levels in F-344 female rats exposed to subchronic oral doses of benzo[a]pyrene. Values are mean ± SD; n = 6–8 animals/dose group; *p < 0.05 (significantly different from controls).](image)

![FIG. 5. Hemoglobin levels in F-344 male rats exposed to subchronic oral doses of benzo[a]pyrene. Values are mean ± SD; n = 6–8 animals/dose group; *p < 0.05 (significantly different from controls).](image)

![FIG. 6. Hemoglobin levels in F-344 female rats exposed to subchronic oral doses of benzo[a]pyrene. Values are mean ± SD; n = 6–8 animals/dose group; *p < 0.05 (significantly different from controls).](image)
a dose-dependent increase in immunosuppression in mice orally treated with 10 to 60 mg BaP/kg. A maximum suppression of 50 to 60% was observed by these authors at a dose of 60 mg/kg. While the exact mechanism for suppression of WBCs or immunosuppression has not been fully elucidated, Trombino et al. (1996) have shown that PAHs induce apoptosis in stromal/feeder cells on which lymphocytes depend for growth or function. BaP and dimethylbenzanthracene (DMBA) at concentrations of $10^{-6}$ M have also induced apoptosis in bone marrow-derived lymphocyte cell lines when they were cultured on a layer of stromal cells (Near et al., 1999). Induction of apoptosis by PAHs such as DMBA in concentrations ranging from $10^{-3}$ to $10^{-1}$ M in murine B-cell lymphoma (Burchiel et al., 1993), and by fluoranthene (0.2 mM) in T-cell hybridomas (Yamaguchi et al., 1999) have also been reported. Szczeklik et al. (1994) suggested that PAHs at concentrations of 0.2–50 μg/m$^3$ could induce immunosuppression in humans exposed to these chemicals through environmental and occupational exposures. Romero et al. (1997) reported that reactive oxidative intermediates of BaP inhibit the proliferation of human peripheral blood mononuclear cell lymphocytes.

Subchronic exposures to BaP also affect other elements of the hematopoietic system, especially the red cell-associated blood parameters. RBC counts (Figs. 1 and 2), Hct (Figs. 3 and 4), and Hgb (Figs. 5 and 6) were respectively decreased a maximum of 10%, 12%, and 12% at doses of 100 mg/kg/day. In a similar oral toxicity study, De Jong et al. (1999) reported a significant decrease in RBC (18%), Hgb (15%) and Hct (14%) in rats at a dose of 90 mg/kg/day. Cruzan et al. (1986) and Feuston et al. (1994) also reported a significant decrease in RBC counts, hemoglobin, hematocrit, and erythroid hypoplasia in bone marrow of rats dermally exposed to doses of 8, 30, 125, and 500 mg/kg clarified slurry oil, of which BaP is a principal component. Severe reductions in these blood elements may lead to anemia, which interferes with oxygen transport to tissues and may induce hypoxia.

The apparent toxicity of BaP appears to be mediated by the induction of the aryl hydrocarbon hydrolase (AHH) enzyme system including CYP1A1 and CYP1A2. Nebert et al. (2000) established through biochemical and molecular studies that the aromatic hydrocarbon receptor (AhR) plays a key role in cell-cycle regulation and apoptosis. AhR activation by PAHs including BaP leads to the induction of AHH, which generate reactive metabolites from the parent compound (by the AhR regulation of AHH enzyme), and which contribute to apoptosis and other cellular damage in biological systems. Twedt et al. (1992) reported that bone-marrow cells in mice exposed to BaP orally activated BaP to the 7,8-diol that requires further activation to the diol epoxide, a reactive form that binds with DNA and induces bone-marrow toxicity. Human erythrocytes metabolically activate BaP to quinone derivatives that are responsible for cytogenetic effects such as induction of sister chromatid exchanges and micronuclei (Lo Jacono et al., 1992). Similarly, human peripheral lymphocytes in cultures exposed to 100 μM BaP were susceptible to genotoxic damage as a result of the metabolites formed by the activation of BaP (Wilson et al., 1995). One DMBA metabolite, the 3,4-dihydrodiol, was reported to induce pre-B-cell apoptosis in bone marrow cultures exposed to DMBA at concentrations ranging from $10^{-8}$ to $10^{-12}$ M (Mann et al., 1999). In addition to apoptosis and cell damage, metabolites have also been implicated in immunotoxicity. Anselstetter and Heimpel (1986) reported a moderate bone marrow depression in BDF1 mice with a decrease in hematopoietic stem cells after acute oral BaP exposure doses of 125 mg/kg. In a subacute study, the number of erythrocytes with micronuclei showed a significant increase in mice that received up to 200 mg/kg BaP orally (Shimada et al., 1990). The aforementioned studies clearly point out that metabolism plays a vital role in BaP toxicity.

In order to address the concern as to whether a sufficient dose of BaP is absorbed into the blood for metabolism, distribution to target tissues, and the subsequent induction of the toxic effects, our team conducted a bioavailability (Ramesh et al., 1999) and toxicokinetic (unpublished data) study of BaP at various time points subsequent to oral administration. This study showed that BaP is present in the plasma at significant levels within 2 h. Along with other metabolites, the 7,8-diol-9,10-epoxide, the ultimate mutagenic metabolite of BaP that causes oxidative damage in target tissues, was also found in plasma. These findings suggest a plausible cause-effect relationship between the parent compound, metabolite levels and types, and the toxic manifestations observed in mammalian tissues.

In our studies, the male animals appeared to be affected more severely by BaP than the female animals. This was especially true in the kidney where 80 and 100% of the male animals at dosages of 50 or 100 mg/kg/day, respectively, developed abnormal tubular casts after 90 days. In contrast,
only 10% of the females developed the abnormality. The casts are molds of the distal nephron lumen and development of casts may be indicative of kidney function abnormalities such as nephrotic syndrome. The intrarenal distribution of cytochrome P450 enzymes might have a role in the development of tubular casts. Endou (1983) reported that P450 was localized only in the proximal tubule of nephrons, whereas the distal tubule possessed no P450. The absence of these enzymes in the distal tubule coupled with the daily exposure to BaP might have affected the distal tubules. Though both sexes were affected, the male animals appeared to be affected more severely. Such male-specific nephrotoxicity (including adenomas and adenocarcinomas) caused by other hydrocarbons has been reported in F-344 rats (Alden, 1986). Chemically induced cytotoxicity as a result of male sex hormone-related α2 microglobulin accumulation in renal tubules was suggested as a causative factor for renal tubule neoplasia in male rats (Hard et al., 1993). Gender-specific differences in the activation of aryl hydrocarbon hydroxylase in rat kidney have also been reported. In kidney cortex microsomes of rats, benzo[a]pyrene 3-hydroxylase was activated by α-naphthoflavone only in females (Benford and Bridges, 1983). Behavioral neurotoxicity studies conducted in our laboratory (Saunders et al., 2001) also indicated that males were more sensitive to BaP toxicity than females when given acute doses up to 200 mg/kg. One possible explanation for the apparent sex difference in outcome could be a difference in the male/female levels of AHH induction by BaP. Ramesh et al. (2000) observed that AHH induction in male and female rats differs, with females showing greater levels. Greater levels of AHH induction may confer some degree of protection or enhance detoxification mechanisms against the effects of BaP. In other words, the higher levels of AHH in females may lead to a more rapid metabolism of BaP and the subsequent excretion of metabolites.

Conversely, the lower levels of AHH in males may contribute to slower metabolism of BaP, increasing the residence time of the parent compound in the body, which, over a period of time, may lead to the formation of reactive metabolites that may elicit toxic effects. Gender-related differences in pharmacokinetics or phase-II detoxification pathways reported by others (Waxman et al., 1985) may account for the differential response to BaP exposure in males and females. The gender-specific biotransformation may result in a different metabolic profile for males and females in terms of type and quantity of metabolites produced. In this context, the findings of Boyle and Craft (2000) are worth mentioning, as these authors reported significant gender-related differences in the rates of diol metabolite formation in hepatic microsomes of Long-Evans and Hooded Lister rats treated with benzo[a]anthracene, a PAH compound. Sierra-Santoyo et al. (2000) reported a several-fold induction of some hepatic P450 isozymes in female rats compared to males treated with DDT, a chlorinated hydrocarbon. In addition to phase-I enzymes, phase-II detoxification such as hepatic glutathione S-transferases were reported to be less inducible in males than females (Chaubey et al., 1994). Another possible alternative for the apparent sex difference may be due to hormonal factors (Bengtsson and Rydstrom, 1973) that may provide some level of protection for the female. However, the apparent protection is not unlimited, since our subchronic data show that the female is ultimately affected at the higher dose levels.

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