Experimental evidence suggests that inorganic lead and benzo[a]pyrene (BaP) suppress the development of primordial oocytes during fetal life. We examined the single and combined effects of prenatal exposure to BaP and moderate doses of lead. The fertility and ovarian morphology of F₁ female NMRI mice in four treatment groups (nine mice per group) were investigated: control; control; lead (F₀ given 1 g PbCl₂/L in drinking water until mating); BaP (10 mg/kg body weight daily by oral intubation on days 7–16 of F₀ pregnancy); and combined lead and BaP. F₁ groups exposed prenatally to BaP either alone or in combination with inorganic lead showed markedly reduced fertility with few ovarian follicles compared to controls, whereas the group exposed to lead only had measures comparable to the controls. Mice exposed to both lead and BaP had a significantly longer gestation period (days to litter) compared to mice exposed only to BaP, lead, or controls. There is a nonsignificant indication that the compounds together further reduce number of offspring, number of litters, and litter size. These results suggest that lead and BaP have synergistic effects on impairment of fertility. The possibility of synergism may be of human relevance as inorganic lead and BaP are ubiquitous environmental pollutants.

Key words: benzo[a]pyrene, fertility, inorganic lead, oogenesis, synergism. Environ Health Perspect 103:588-590 (1995)

The period of development of the female gonads in fetal life may be critical for fertility in adult life. Rodents show a reduced number of primordial oocytes or reduced fertility after prenatal exposure to inorganic lead (1–3), benzo[a]pyrene (BaP) (4), and other agents (5,6). Inorganic lead is also a suspected developmental neurotoxicant (7) and could possibly have adverse effects on the developing reproductive system through action on the hypothalamic-pituitary axis during fetal life. Studies exploring if similar mechanisms may be responsible for human subfecundity are scarce; in an epidemiologic study maternal smoking in the pregnancy was associated with reduced fecundability among their daughters in later life (8).

The reproductive effects of environmental pollutants are currently receiving attention, due in part to the possibility of human infertility (9). BaP and inorganic lead are of special interest. Both are ubiquitous environmental pollutants, and BaP is a component of cigarette smoke. We therefore conducted an experiment exposing mice prenatally to inorganic lead and BaP. The purpose of the study was to investigate lead and BaP for synergistic effects on female fertility. We chose a treatment dose of lead that was judged from the blood lead levels to be comparable to exposure levels found in many occupational settings. The treatment dose of BaP was similar to the lowest dose that induced subfertility in an earlier report (4).

Methods

Lead(II) chloride (CAS no. 7758-95-4) was supplied from Baker (Deventer, Holland). Benzo[a]pyrene (CAS no. 50-32-8) was a product of Sigma Chemical Company (St. Louis, Missouri).

Male and F₀ female Bom:NMRI mice were acclimatized to a 12/12 hr light/dark cycle (lights on at 0600 hr) at 24 ± 1°C. Laboratory chow (EWOS, R34) and tap water were provided ad libitum. Males were individually caged except during mating. We placed F₀ females in groups of three until the last week of pregnancy when they were caged alone.

At the age of 9 weeks, F₁ females were randomly assigned to one of four treatment groups, nine mice per group. Two groups received tap water with 1 g PbCl₂/L(0.75 g Pb) during the 6 weeks before mating, whereas the remaining two groups received tap water without any additions. We discontinued the lead treatment before mating to avoid exposure of the males. All F₁ females were caged with sexually active males. Females were inspected twice daily for a vaginal plug, which, in case of pregnancy, was counted as day 0 of gestation. During days 7–16 of pregnancy, one of the tap water groups and one of the lead–water groups received a daily treatment of 0.2 mL corn oil BaP (10 mg/kg body weight by oral intubation), and the remaining two groups received corn oil. Thus, the four treatment groups, each comprising nine F₁ females, were the control group, the lead group, the BaP group, and the lead plus BaP group. We recorded weights and sampled blood for lead measurement from tail veins on the day before caging with the males (Table 1). Assessed from a pilot trial on female mice of the same strain, the blood lead values in the lead-treated groups should give values of approximately 2 μmol/L 10 days later (during the second week of pregnancy).

No F₂ females showed signs of general toxicity, and they all proved fertile. We kept F₂ females with their offspring until after weaning (21 days after delivery).

One F₁ female from each of the 36 litters was allocated in the experiment, each belonging to one of the four exposure groups they had been assigned to in utero. At the age of 6 weeks, each F₁ female was caged for 6 months with an untreated male proven to be sexually active. Males for four females that did not become pregnant after 30 days were replaced, but this procedure did not lead to any pregnancies. Dates and sizes of litters were recorded. F₂ offspring were inspected for gross deformities at birth, and their weight and sex were recorded at day 2 after birth when they were killed by cervical dislocation.

After 6 months of continuous breeding, the F₁ females were euthanized; the right ovary was excised, trimmed, and weighed. Ovaries were fixed in buffered formalin, embedded in paraffin, and 3-μm parasagittal sections were prepared. Three slides, made from tissue 90 μm apart, were stained with hematoxylin–azophloxine-safron and reticulin and examined by light microscopy.

The F₁ female was taken as the basic unit of experimentation. Several measures of fertility and ovarian morphology were recorded without knowledge of the treatment group. Measures of fertility for each F₁ female were number of offspring, number of litters during the breeding period, median litter size, and median days between deliveries (with the start of the experiment as the starting point for the first interval).

We recorded the weight of the right ovary for each F₁ female and counted follicles and corpora lutea in the three histological sections. Follicles were classified according to Pedersen and Peters (10).

Treatment effects on fertility, ovarian weights, and counts in the microscopic examination were evaluated by nonparametric tests (Wilcoxon rank sum test, Kruskall–Wallis test). Significance levels of <0.05 (two-tailed) were taken as criterion of treatment effects. The statistical analyses were performed with the BMDP software package (11).

Results

The 36 mated F₁ females gave birth to a total of 1985 offspring distributed over 182 litters. The distribution over the treat-
Table 1. Descriptive values (median, range) for F2 and F1 female mice in the four treatment groups

| Treatment group | F2 females (n = 9/group) | F1 females (n = 9/group) |
|-----------------|--------------------------|--------------------------|
|                 | Body weight at time of mating (g) | Blood lead at time of mating (µmol/L) | Body weight after first pregnancy (g) |
| Control         | Median 37.8 Range 34.6–41.4 | Median 0.04 Range 0.01–0.10 | Median 38.7 Range 37.3–43.9 |
| Lead            | Median 36.5 Range 33.3–43.9 | Median 3.37 Range 2.44–3.80 | Median 39.8 Range 37.9–44.7 |
| BaP             | Median 35.9 Range 32.8–40.3 | Median 0.04 Range 0.02–0.08 | Median 37.1 Range 34.1–44.3 |
| Lead plus BaP   | Median 37.9 Range 32.7–46.3 | Median 3.71 Range 2.70–5.26 | Median 38.0 Range 32.9–44.7 |
| Heterogeneity between groups, χ² (p) | 0.33 (0.82) | 27.3 (<0.0001) | 1.98 (0.58) |

BaP, benzo[α]pyrene.
*Significantly different from control group (two-tailed p < 0.05).
**Significantly different from control group (two-tailed p < 0.005).

Table 2. Values of different measures of fertility over the four treatment groups among F1 female mice

| Effect | Control (n = 9) | Lead (n = 9) | BaP (n = 9) | Lead plus BaP (n = 9) | Heterogeneity between groups, χ² (p) |
|--------|----------------|-------------|-------------|-----------------------|-------------------------------------|
| No. of litters | 67 (9)         | 72 (8)      | 29 (8)      | 14 (9)                |                                    |
| No. of offspring (stillborn) | 785 (4)       | 860 (8)    | 248 (8)    | 92 (9)                |                                    |
| Median no. offspring/F1 (range) | 92 (26–121)   | 95 (50–122)| 22** (0–86)| 4** (0–48)            | 24.5 (<0.0001)                     |
| Median no. litters/F1 (range) | 8 (3–8)       | 8 (0–8)    | 3** (0–8)  | 2** (0–5)             | 22.8 (<0.0001)                     |
| Median litter size/F1 (range) | 11.5 (6–15)   | 13 (6–16)  | 8** (3–11) | 6.5** (1–12)          | 10.6 (0.01)                        |
| Median no. days between litters/F1 (range) | 20.5 (20–21)  | 20.5 (20–21)| 21* (20–23) | 22.5** (21–29)        | 15.5 (0.001)                       |

BaP, benzo[α]pyrene.
*Chi-square with 3 degrees of freedom; two-tailed p-value.
**Significantly different from control group (two-tailed p < 0.05).

The Kruskall-Wallis test demonstrated highly significant heterogeneity over the treatment group for all measures of fertility: number of offspring, number of litters, median number of offspring per litter, and days between litters (Table 2). This was due to markedly lower fertility measures in the BaP and the lead plus BaP groups compared to the control group, whereas the lead group performed nonsignificantly better than the control group for all measures. In the comparison between the BaP group and the lead plus BaP group, the latter showed poorer fertility for all measures. Some of those differences were large but were only significant for median days between litters (Z-value = 1.98, two-tailed p = 0.05). For other effect measures, the significance levels ranged from 0.09 to 0.32.

A similar pattern was evident from examination of the ovaries (Table 3). Ovary weights, counts of follicles, and counts of corpora lutea were different in the treatment groups, with low weights and depletion of follicles and corpora lutea for most animals in the BaP and lead plus BaP groups. The F1 females in the lead group had nonsignificantly higher weights and counts of follicles and corpora lutea compared to the control group. The lead plus BaP group had lower counts of follicles and corpora lutea and slightly higher median ovarian weight than the BaP group, but all differences were nonsignificant in the Wilcoxon rank sum test.

Discussion

We have shown that prenatal administration of 10 mg BaP/kg maternal body weight (with or without lead) leads to subfertility and a marked reduction in the number of ovarian follicles in F1 females. These results are in agreement with a previous report (4) and are probably due to impaired development of primordial oocytes. This interpretation is further supported by an earlier report on toxic effects of BaP on primordial oocytes in weaning mice (12).

No treatment effects of lead were detected in the study. This is not surprising since we used moderate doses resulting in maternal blood lead levels about 2 µmol/L during the second week of pregnancy; this is considered to be the critical time of fetal folliculogenesis (3). This dose was far lower than that reported to have an independent effect on primordial germ cells (3) and fertility (1–3).

To our knowledge, the combined effect on fertility of inorganic lead and BaP has not been reported earlier. Our study was designed to investigate the hypothesis of an interaction between the two agents. We found a synergistic action of inorganic lead on the BaP effects for days between litters, and the manifestations of combined lead and BaP treatment were stronger, but not
significantly so, for almost all indicators of impaired follicular development and fertility compared to the BaP treatment alone. It may be relevant that some lead compounds have synergistic effects on model carcinogens in rodents (13–16).

Mechanistic interpretations on the synergism between lead and BaP on the basis of our results can only be speculative. BaP seems to have a direct effect on primordial oocytes (4,7). Fetal treatment with high doses of inorganic lead also decreases the number of primordial follicles, possibly as an effect on the migration or multiplication of the developing germ cells (2). Lead is also a developmental neurotoxicant, and its synergistic action might be explained by a disturbance of the neuroendocrine balance that alone was not sufficient to impair fertility. However, Wide (2) found only small and nonsignificant alterations in the levels of ovarian steroid hormones for mice exposed prenatally to lead in doses that clearly reduced the number of primordial follicles.

The lead and BaP doses chosen in our study were probably not optimal. The lead dose was comparable to human exposures, and the daily BaP dose in our study was at the lowest effect level in an earlier study, but its effect on fertility was strong (4). The daily dose (10 mg/kg) is about 1 million times the main stream dose in 100 cigarettes (17). Fertility as outcome might have been more sensitive to the combined effects of lead and BaP if the BaP dose had been lower. BaP alone had a profound effect; even if the lead plus BaP group had almost total depletion of follicles and several animals were infertile, the differences were not significant.

There is currently much concern about human fertility (9). Biology indicates that the prenatal development of primordial germ cells may be crucial. Exposures to common environmental xenobiotics are under suspicion of interfering with gonadal development (9). Human exposures from environmental sources are considerably lower than exposures producing effects in laboratory animals but could be more relevant in case of synergistic actions. Human studies addressing effects of environmental agents are warranted, but the needed multigenerational designs restrict the possibilities (18). Animal models that have been developed should be further applied.

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