Prenatal Effects of Caffeine and Restraint Stress in Mice (44352)

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Abstract. **The maternal and developmental toxicity of combined exposure to restraint stress and caffeine was assessed in mice. On Day 9 of gestation, six groups of pregnant mice were treated (p.o.) with a single dose of 30, 60, or 120 mg/kg of caffeine. Immediately after caffeine administration, three of these groups were subjected to restraint for 14 hr. Control groups included unrestrained and restrained pregnant mice not exposed to caffeine. An additional group of animals (unrestrained and not exposed to caffeine) was deprived of food for 14 hr. A two-way (caffeine dose x restraint) analysis of variance revealed an overall effect (reduction) of restraint and caffeine exposure on maternal body weight gain and food consumption on gestation Days 9–11. Significant reductions were also observed in body weight at termination and corrected body weight change of dams concurrently exposed to 120 mg/kg of caffeine and restraint. By contrast, no significant effects of caffeine, restraint, or caffeine plus restraint on embryo/fetal development were noted. The doses of caffeine administered here are much higher than those usually consumed by the general population. Under the current experimental conditions, caffeine alone or combined with restraint stress was not embryotoxic or teratogenic in mice.** [P.S.E.B.M. 1999, Vol 220]

Caffeine(1,3,7-trimethylxanthine), a stimulant drug, is present in a great variety of products, such as coffee, tea, chocolate, cola beverages, and some medications, which are widely consumed by the general population even during gestational periods. Although it has been reported that caffeine does not have teratogenic effects on the developing human fetus when intake is moderate and spread out over the day, this drug can enhance the teratogenic effects of other substances (1–3). Although caffeine is well known to potentiate the cytotoxic actions of radiation, alkylating agents and other teratogenic agents in chicken, rats, and mice, no interactions between caffeine intake and either alcohol consumption or smoking during pregnancy have been demonstrated in either animals or humans (3). In relation to the potential interactions between physical and chemical agents, it should be taken into account that although many human birth defects can be attributed to single mutant genes, chromosomal abnormalities, environmental effects, or multifactorial interactions, a remarkable number of birth defects are still of unknown etiology.

In contrast to human exposure, the teratogenic effect of caffeine has been clearly shown in rodents, with mice being more sensitive than rats (1). However, the teratogenic effect of caffeine in these species appears usually at high doses that are also toxic to the dam (1, 4).

On the other hand, maternal stress during gestation can produce significant fetal and/or postnatal effects and can enhance the teratogenicity of a number of chemicals (5–11). It has been demonstrated that maternal stress induced by heat, cold, noise, visual stimuli, or immobilization is capable of adversely affecting the developing conceptus (7, 11–13). Among the experimental models to cause maternal stress, restraint often has been used in rodents as an archetype easily controlled stressor that imposes both physical and psychological demands on the subject (11, 14, 15). Maternal restraint stress in mammals has been associated with adverse effects on implantations, embryo/fetal viability, lowered birth weight of the offspring, skeletal malformations and neurobehavioral changes (5, 11, 16–18). These effects depend on the degree, type and duration of restraint, as well as the day(s) of gestation when stress is applied (6, 11, 14, 19).

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Although in recent years a number of coteratogenicity studies have been carried out using various physical and chemical agents along with caffeine (3), the effect of stress on the response to caffeine has not been examined to any great extent. While it has been shown in rats that prenatal stress exposure (pregnant animals were handled 5 min daily from the 14th to 21st day of gestation) decreased the effect of caffeine (10 or 30 mg/kg in a single i.p. injection) in the adult offspring on gnawing behavior in an open field (20), there is not information on the influence of maternal stress on the teratogenic potential of this stimulant drug. Pregnant women may be exposed to various types of stress, either in home or in the workplace, while they can also ingest caffeine containing products during gestation. Because both maternal stress and caffeine have been reported to produce developmental toxicity in mammals, the aim of the current study was to determine whether exposure to caffeine during gestation in combination with maternal restraint could result in interactive effects in the mouse.

Materials and Methods

Animals. Mature male and female Swiss Mice (Interfauna Ibérica, Barcelona, Spain) weighing 28–32 g were used. After a quarantine period of 7 days, female mice were mated with males (2:1) overnight and examined the following morning for copulatory plugs. The day on which a vaginal plug was found was designated Day 0 of gestation. Animals were housed five per cage (polyethylene) and kept in an animal room (temperature 22 +/– 2°C, relative humidity of 50 +/– 10%, and automatic light cycle: 0800–2200 hr), with free access to food (Panlab rodent chow, Barcelona) and tap water.

Drug. Caffeine was purchased from Sigma Chemical Co. (St. Louis, MO). It was dissolved in deionized water and administered at doses of 0, 30, 60, and 120 mg/kg.

Procedure. On the evening of gestational Day 9, pregnant mice were weighed and randomly assigned to one of nine groups. A control group was unrestrained and received deionized water by gavage. A second group of animals (restrained control group) was immobilized for 14 hr by placing the pregnant mice in stainless steel cylindrical holders that were lined with foam padding. The restrained mice were held in a prone position with the paws immobilized with elastic adhesive tape. According to previous studies, this procedure produces stress in the pregnant mouse (5, 7, 16). A third group of pregnant mice was also unrestrained but was deprived of food for 14 hr. Animals in the remaining six groups received a single oral dose of caffeine at 30, 60, or 120 mg/kg. Three of these groups were unrestrained whereas the other three groups were subjected to restraint for 14 hr. On the morning of gestational Day 10, animals were again housed in the cages, and food and water were available *ad libitum*.

Data Collection. During the period of gestation, maternal body weight and food consumption were measured daily. On gestation Day 18, animals were sacrificed with diethyl ether, and the number of total implants, resorptions, and live and dead fetuses were recorded. All live fetuses were dissected from the uterus and evaluated for sex, body weight, and external and gross malformations. Approximately one-half of the available fetuses were fixed in 95% ethanol, cleared with 1% KOH, stained with Alizarin red S, and examined for skeletal malformations and variations (20). The remaining fetuses were fixed in Bouin's fluid, sectioned, and evaluated for internal abnormalities (21).

Data Analysis. The unit of comparison was the pregnant female or the litter. Results were computed by a two-way (caffeine dose × restraint) analysis of variance (ANOVA). *Post hoc* analysis consisted of an LSD (least significant difference) method for multiple comparisons between groups when appropriate. The incidence of fetal anomalies between litters was analyzed by means of a two-tailed Fisher exact probability test for pair-wise comparison of groups. Significance was set at the 0.05 probability level.

Results

Since the unrestrained/no caffeine group did not differ from the food deprivation/no caffeine group on any of the dependent variables, both groups were combined in a new unrestrained/no caffeine group.

Maternal Toxicity. The effects of maternal restraint and caffeine on body weight gain and food consumption are shown in Table I. On gestation Days 9–11, maternal body weight gain was significantly lower in the groups exposed to caffeine plus restraint than in the unrestrained control group or in the group of animals deprived of food for 14 hr. However, no differences with the groups treated with caffeine only, or with the groups receiving a combined administration of caffeine and restraint were observed. Moreover, a significant reduction in maternal weight gain was also noted on gestation Days 9–11 in animals subjected to restraint only.

A two-way (caffeine dose × restraint) analysis of variance revealed an overall effect of restraint and caffeine exposure on maternal body weight gain on gestation Days 9–11 ($F[1,91] = 104.7$, $P < 0.001$; $F[3,91] = 16.0$, $P < 0.001$). A significant interaction restraint × caffeine ($F[3,91] = 4.79$, $P < 0.004$) was also observed. In turn, the differences between groups found on gestation Days 12–15 could be attributed to the action of restraint stress ($F[1,91] = 8.93$, $P < 0.004$), or the combined effects of caffeine plus restraint ($F[4,91] = 3.24$, $P < 0.016$). Although on late gestation (Days 16–18), no significant effects of restraint and caffeine were found ($F[1,94] = 2.87$, $P < 0.094$; $F[3,94] = 2.66$, $P < 0.053$), the combined effects of both (sum of simple effects) were significant ($F[4,94] = 2.84$, $P < 0.029$). On the other hand, differences in total body weight gain (Days 0–18) could be explained by the influence of caffeine ($F[3,94] = 4.49$, $P < 0.006$), or the combined effects of caffeine and restraint ($F[4,94] = 4.19$, $P < 0.004$).

Compared to the control groups, reductions in food

Table I. Effects of Caffeine, Restraint, and Caffeine plus Restraint on Maternal Body Weight Gain and Food Consumption in Pregnant Mice

Caffeine (mg/kg) Type of stress	0 None	30 None	60 None	120 None	0 Restraint	30 Restraint	60 Restraint	120 Restraint
Number of dams	9	11	12	10	25	10	9	10
Gestation days				Body weight gain (g)				
0–8	6.31 ± 0.92	6.32 ± 0.86	6.47 ± 1.46	5.55 ± 1.71	6.00 ± 1.44	6.67 ± 1.70	6.89 ± 1.06	5.67 ± 1.57
9–11	2.84 ± 0.65^{a}	1.65 ± 2.25^{ac}	1.59 ± 0.98^{ac}	1.08 ± 1.17^{c}	0.84 ± 2.41^{dc}	−3.10 ± 1.70^{b}	−3.15 ± 1.45^{b}	−2.34 ± 1.95^{b}
12–15	11.26 ± 3.06^{ac}	10.13 ± 2.95^{ac}	8.70 ± 2.40^{c}	10.17 ± 3.22^{ac}	12.00 ± 4.36^{ab}	15.02 ± 6.32^{b}	13.13 ± 4.85^{ab}	10.37 ± 4.81^{ac}
16–18	10.46 ± 3.25	11.52 ± 3.90	11.13 ± 3.63	8.16 ± 1.25	8.78 ± 4.07	9.18 ± 5.56	10.59 ± 3.31	7.39 ± 3.12
0–18	30.87 ± 2.55^{a}	29.62 ± 6.06^{ac}	27.88 ± 4.91^{ac}	24.96 ± 5.00^{bc}	27.61 ± 5.69^{ac}	27.77 ± 8.33^{ac}	27.46 ± 7.32^{ab}	21.09 ± 6.75^{b}
				Food consumption (g)				
0–8	50.87 ± 3.55	56.92 ± 9.71	51.28 ± 6.86	56.25 ± 7.95	48.89 ± 4.95	54.32 ± 3.64	51.20 ± 9.74	53.2 ± 16.44
9–11	9.25 ± 1.58^{a}	6.94 ± 1.52^{a}	7.94 ± 1.58^{a}	7.22 ± 1.18^{a}	8.47 ± 3.37^{a}	2.03 ± 1.84^{b}	2.43 ± 1.46^{b}	1.11 ± 0.47^{b}
12–15	25.16 ± 1.65	26.85 ± 1.73	25.47 ± 2.04	30.52 ± 4.88	27.28 ± 2.49	29.50 ± 1.74	28.93 ± 1.69	27.37 ± 3.50
16–18	28.33 ± 3.11^{a}	34.10 ± 15.91^{ad}	28.65 ± 5.97^{ad}	17.17 ± 3.89^{bc}	26.03 ± 3.47^{bcd}	24.73 ± 3.48^{bcd}	25.55 ± 2.88^{bcd}	17.08 ± 2.64^{bc}
0–18	114.30 ± 12.74	119.9 ± 20.18	113.35 ± 2.94	110.95 ± 7.57	114.21 ± 10.77	115.98 ± 12.74	108.10 ± 20.17	91.91 ± 22.70

Note. Values are given as means ± SD. Results showing different superscripts (^{a,b,c,d}) indicate significant differences at $P < 0.05$.

consumption were also observed on gestation Days 9–11. Mice in the groups concurrently exposed to caffeine and stress ingested lower amounts of food than those in the remaining groups (Table I). Reductions were also statistically significant when compared with the groups exposed only to caffeine or restraint. A two-way (caffeine dose × restraint) analysis of variance showed an overall effect of restraint and caffeine exposure on maternal food consumption on gestation Days 9–11 ($F[1,34] = 4.16$, $P < 0.001$; $F[3,34] = 11.76$, $P < 0.001$), whereas a significant interaction restraint × caffeine dose was also noted ($F[3,34] = 3.64$, $P < 0.025$). These effects were also statistically significant on late gestation (Days 16–18) ($F[1,33] = 6.83$, $P < 0.015$; $F[3,33] = 7.54$, $P < 0.001$). However, no significant effects of either caffeine or restraint were noted on total food consumption (Days 0–18). On gestational Days 9–11 and 16–18, significant differences between groups might have been due to the effects of restraint ($F[1,33] = 5.24$, $P < 0.03$), caffeine ($F[3,33] = 3.66$, $P < 0.025$), or their combined effects ($F[4,34] = 3.71$, $P < 0.016$).

Table II summarizes the effects of caffeine and restraint stress on a number of maternal variables. Only body weight at termination and corrected body weight change (corrected body weight minus body weight on Day 0 of gestation) showed significant differences among groups. As in maternal body weight gain, the most remarkable reductions in both variables were observed in dams concurrently exposed to 120 mg/kg of caffeine and restraint. However, the differences were not statistically significant when compared with the unrestrained and restrained control groups or with the groups given caffeine only. The differences between groups on body weight at termination could be attributed to the effects of the caffeine dose ($F[3,93] = 4.91$, $P < 0.003$), whereas the differences between groups on corrected body weight change could be due to the effects of restraint, caffeine, or the combined effects of both ($F[1,88] = 7.26$, $P < 0.09$; $F[3,88] = 3.81$, $P < 0.013$; $F[4,88] = 4.27$, $P < 0.003$).

Embryo/Fetal Toxicity. Data on pregnancy outcome measures are shown in Table III. There were no significant differences among groups in the number of total implants per litter, the number of viable and nonviable implants per litter, or in the sex ratio. However, fetuses in the groups whose mothers were exposed to 60 and 120 mg/kg of caffeine plus restraint showed a lower mean body weight than the control groups or the group given caffeine only. This reduction could be attributed to the effects of restraint ($F[1,90] = 5.35$, $P < 0.023$) or to the combined effects of restraint and caffeine ($F[4,90] = 2.77$, $P < 0.032$). No interactions between caffeine and stress could be observed in any of the different parameters evaluated.

On the other hand, no external, internal or skeletal malformations, which could be attributed to caffeine or restraint were found. However, a two-way (restraint × caffeine dose) analysis of variance revealed an overall effect of restraint and/or caffeine on the ossification of the parietal (restraint: $F[1,90] = 21.63$, $P < 0.001$; caffeine: $F[3,90] = 30.6$, $P < 0.001$; interaction: $F[3,90] = 12.38$, $P < 0.001$) and occipital bones (restraint: $F[1,90] = 11.89$, $P < 0.001$; caffeine: $F[3,90] = 8.76$, $P < 0.001$), sternum (interaction: $F[3,90] = 4.88$, $P < 0.004$), xiphoid (caffeine: $F[3,90] = 10.93$, $P < 0.001$), and caudal vertebrae (restraint: $F[1,90] = 21.10$, $P < 0.001$; caffeine: $F[3,90] = 51.10$, $P < 0.001$; interaction: $F[3,90] = 20.69$, $P < 0.001$). By contrast, no significant effects in the total decreased ossification among groups were found (Table IV).

Discussion

World coffee consumption is increasing. The daily intake of caffeine in the general population has been reported to range from 202–283 mg/day, which would represent about 2.7–4.0 mg/kg/day in males and females between 20 and 75 years old (1). Given the relatively large quantities of caffeine that are needed to induce malformations, which in turn affect a relatively small number of animals, this drug has been considered as a weak teratogenic agent (1). It has been reported that mice are more sensitive than rats to the developmental effects of caffeine (1). Whereas rats malformations are rarely observed for doses less than 80–100 mg/kg/day given by gavage or i.p. injection, abnormalities in

Table II. Effects of Caffeine, Restraint, and Caffeine plus Restraint on some Maternal Variables in Pregnant Mice

Caffeine (mg/kg)	0	30	60	120	0	30	60	120
Type of stress	None	None	None	None	Restraint	Restraint	Restraint	Restraint
Number of dams	9	11	12	10	11	10	9	10
Body weight at termination (g)	60.35 ± 6.33[a]	58.17 ± 7.27[ac]	56.90 ± 4.72[ab]	53.06 ± 5.10[cb]	58.12 ± 4.73[a]	56.30 ± 8.63[ab]	56.13 ± 7.63[ab]	50.84 ± 7.84[b]
Gravid uterine weight (g)	20.33 ± 4.39	19.11 ± 5.40	18.61 ± 3.94	16.80 ± 4.78	19.96 ± 4.29	19.16 ± 7.66	18.91 ± 4.72	17.52 ± 4.80
Corrected body weight (g)	41.20 ± 3.05	39.06 ± 2.53	38.29 ± 2.53	36.26 ± 1.46	38.10 ± 2.83	37.14 ± 10.70	37.22 ± 3.11	35.04 ± 2.42
Corrected body weight change (g)	9.92 ± 1.20[ad]	10.52 ± 1.43[a]	9.27 ± 2.12[ac]	8.16 ± 1.65[cd]	9.46 ± 1.97[ad]	8.08 ± 6.05[dc]	7.93 ± 3.10[dc]	5.91 ± 2.47[bd]
Liver weight (g)	2.68 ± 0.30	2.65 ± 0.29	2.62 ± 0.29	2.49 ± 0.23	2.59 ± 0.23	2.54 ± 0.25	2.53 ± 0.34	2.35 ± 0.33
Relative liver weight (%)	4.53 ± 0.38	4.57 ± 0.42	0.48 ± 0.10	4.71 ± 0.44	4.48 ± 0.44	4.66 ± 1.22	0.44 ± 0.05	4.64 ± 0.30
Kidney weight (g)	0.47 ± 0.05	0.48 ± 0.05	0.85 ± 0.14	0.44 ± 0.04	0.46 ± 0.05	0.44 ± 0.04	0.79 ± 0.15	0.43 ± 0.06
Relative kidey weight (%)	0.80 ± 0.11	0.83 ± 0.13	0.85 ± 0.14	0.83 ± 0.06	0.80 ± 0.09	0.81 ± 0.20	0.79 ± 0.15	0.85 ± 0.12

Note. Values are given as means ± SD. [a,b,c,d]Results showing different superscripts indicate significant differences at $P < 0.05$.

mice have been found to occur at oral or parenteral doses of 50–75 mg/kg/day of caffeine (1, 22). The most frequently observed malformations in rats and mice are those of the limbs and digits, ectrodactyly, craniofacial malformations, and delays in ossification of limbs, jaw, and sternum (1).

Despite the low teratogenic potential of caffeine in mammals, this drug can potentiate the effects of various teratogenic agents such as nicotine (23), ionizing radiations (24, 25), or a variety of pharmaceutical compounds (acetazolamide, mytomycin C, hydroxyurea, 5-fluorouracil) (2, 3, 26, 27). It should be remembered that some potentiative interactions among physical and chemical agents could be one of the causes of human malformations of uncertain origin (1, 28). Results of the literature review suggest that, in humans, heavy caffeine consumption (>300 mg/day) during pregnancy is associated with small reductions in infant birth weight that may be especially detrimental to premature or low-birth-weight infants (29). An increased risk of spontaneous abortion associated with caffeine consumption prior to and during pregnancy also has been documented (30, 31).

On the other hand, it has been shown that behavioral and physiological stress during gestation, such as conditioned anxiety, crowding, immobilization, or temperature extremes, permanently modifies structural or functional development of offspring in rats and mice (17, 32–34). In the current study, we assessed in mice whether maternal-restraint stress could potentiate the maternal and developmental effects of caffeine in pregnant mice.

Restraint for 14 hr, starting in the evening of Day 9 of gestation, was used as a "stressor" for the induction of maternal stress, whereas the doses of caffeine were based on previous studies on the potential teratogenicity of this drug in mice (3, 26). Paré and Glavin (14) reviewed the use of restraint in experimental biomedical research. They reported that immobilization of the animal for at least 1–5 hr is associated with a variety of both central and peripheral changes indicative of stress. Although it was noted that there was some variability in the results depending on the duration of restraint, the majority of studies showed that increasing the duration increased the degree of stress pathology observed (1). According to previous studies in our laboratory, the current procedure causes stress in pregnant mice (7, 9). On the other hand, in relation to the day and doses of caffeine administration, a notable number of coteratogenicity studies have been carried out using various physical and chemical agents along with caffeine (3). In those studies, the caffeine dose was remarkably variable, as well as the time of administration, which ranged from the 7th to the 13th day of gestation. In the present study, the choice of gestation Day 9 as the caffeine treatment day was based on a previous investigation on the effects of caffeine on the teratogenicity of acetazolamide (26).

Elmazar *et al.* (4) administered caffeine to pregnant mice at doses up to 250 mg/kg/day in drinking water (gestation Days 5–18) or up to 150 mg/kg/day in pellets (gestation Days 5–18). Apart from a low incidence of cleft

Table III. Effects of Caffeine, Restraint, and Caffeine plus Restraint on Reproductive Findings

Caffeine (mg/kg) Type of stress	0 None	30 None	60 None	120 None	0 Restraint	30 Restraint	60 Restraint	120 Restraint
Number of litters	9	11	12	10	24	10	9	9
Implantation/litter	11.78 ± 3.38	12.09 ± 2.98	12.00 ± 2.70	10.30 ± 3.30	12.71 ± 2.63	11.54 ± 3.42	13.11 ± 2.42	11.11 ± 3.37
Live fetuses/litter	10.89 ± 3.40	11.54 ± 3.41	11.08 ± 2.75	9.60 ± 2.95	11.71 ± 2.37	11.40 ± 5.42	11.67 ± 2.45	10.89 ± 3.37
Dead fetuses/litter	0.11 ± 0.33	0.00 ± 0.00	0.17 ± 0.39	0.00 ± 0.00	0.25 ± 0.44	0.40 ± 0.51	0.33 ± 0.71	0.00 ± 0.00
Early resorptions/litter	0.56 ± 0.73	0.45 ± 0.69	0.17 ± 0.39	0.40 ± 0.70	0.50 ± 1.02	0.10 ± 0.31	1.11 ± 1.97	0.00 ± 0.00
Late resorptions/litter	0.22 ± 0.44	0.09 ± 0.30	0.58 ± 0.90	0.30 ± 0.67	0.25 ± 0.44	1.00 ± 2.82	0.00 ± 0.00	0.22 ± 0.4
Postimplantation loss litter (%)	7.47 ± 8.42	5.55 ± 7.09	7.54 ± 9.24	6.47 ± 6.61	8.68 ± 9.89	14.24 ± 30.50	10.18 ± 13.00	1.85 ± 3.77
Mean fetal weight/litter (g)	1.30 ± 0.06^{a}	1.29 ± 0.05^{a}	1.27 ± 0.10^{a}	1.29 ± 0.08^{a}	1.27 ± 0.08^{a}	1.30 ± 0.21^{a}	1.22 ± 0.09^{ab}	1.16 ± 0.10^{b}
Fetal sex ratio (M/M+F)	0.49 ± 0.15	0.51 ± 0.11	0.49 ± 0.13	0.54 ± 0.18	0.52 ± 0.15	0.47 ± 0.15	0.49 ± 0.11	0.45 ± 0.12

Note. Values are given as means ± SD.

palate, no gross abnormalities that were attributable to caffeine treatment were observed. The most important effect observed was a reduction in fetal body weight. Retarded ossification (particularly of the suppraoccipital bone) was also observed in fetuses when caffeine (150 mg/kg) was administered in the drinking water but not when the same dose was given as a sustained release pellet (4). On the other hand, caffeine injected s.c. at 50 mg/kg to mice on Day 11 of gestation did not affect the normal embryonic development as compared to the group of animals treated with saline s.c. and i.p. (control groups) (10). In turn, although a dose of 50 mg/kg of caffeine injected s.c. to pregnant mice on Day 9 of gestation was not itself teratogenic, it significantly enhanced the rate and severity of fetal malformations in animals exposed to 200 or 1000 mg/kg of acetazolamide (26). The frequency and severity of acetazolamide-induced ectrodactyly were potentiated by caffeine, whereas the reduction of the number of ossified cervical and caudal vertebral central among litters exposed to acetazolamide was also augmented by co-administration of caffeine.

The results of this study show a slight effect of caffeine and restraint stress on some maternal parameters. Compared to the control group, body weight gain on gestation Days 9–11 was lower in all restrained groups, whereas weight gain of dams in the groups exposed concurrently to caffeine and restraint was lower than that in the groups given caffeine only ($P > 0.05$). A similar finding was also seen for food consumption in the groups concurrently exposed to caffeine and restraint, as well as in body weight at termination and corrected body weight change in the group given 120 mg caffeine/kg plus restraint. Taking into account the body weight gain and food consumption observed in the group deprived of food for 14 hr, the present maternal effects would not be attributable to food deprivation.

In contrast to the maternal findings, no significant effects of caffeine, restraint stress, or caffeine plus restraint on embryo/fetal development were noted. Reduced fetal weight together with some delays in fetal ossification were the most notable anomalies observed in the groups concurrently exposed to 120 mg caffeine/kg plus restraint. In addition to the reduction in fetal body weight and the delays in fetal ossification, ectrodactyly and cleft palate were also found in previous investigations on the teratogenic effects of caffeine in mice (4, 35, 36). However, these abnormalities were not observed in the current study.

Maternal restraint stress has been reported to potentiate the effects of some teratogenic agents such as salicylate (37), arsenate (10), or methylmercury (7). By contrast, ma-

Table IV. Effects of Caffeine, Restraint, and Caffeine plus Restraint on Morphological Defects in Mouse Fetuses

Dose of caffeine (mg/kg) Type of stress	0 None	30 None	60 None	120 None	0 Restraint	30 Restraint	60 Restraint	120 Restraint
				Skeletal exam				
Number of litters examined skeletally (fetuses)	9 (52)	10 (63)	12 (76)	9 (45)	23 (141)	10 (67)	9 (67)	9 (51)
Total bone retardations	9 (23)	10 (30)	10 (49)	9 (27)	19 (69)	10 (70)	9 (39)	8 (38)
parietal	1 (2)^{a}	2 (2)^{ab}	5 (10)^{ab}	9 (18)^{bc}	3 (6)^{ab}	10 (40)^{bc}	7 (17)^{bc}	8 (21)^{ab}
occipital	0 (0)^{a}	2 (2)^{a}	1 (1)^{a}	4 (8)^{a}	2 (5)^{a}	7 (30)^{a}	3 (8)^{a}	7 (10)^{a}
sternum	6 (11)^{a}	4 (5)^{a}	4 (5)^{a}	2 (2)^{a}	11 (13)^{a}	6 (22)^{a}	6 (13)^{a}	2 (2)^{a}
xiphoid bipartite	0 (0)^{a}	6 (11)^{ab}	3 (4)^{ab}	3 (4)^{ab}	3 (3)^{ab}	9 (30)^{b}	3 (8)^{ab}	2 (2)^{ab}
vertebrae	0 (0)^{a}	0 (0)^{a}	0 (0)^{a}	0 (0)^{a}	0 (0)^{a}	2 (3)^{a}	0 (0)^{a}	0 (0)^{a}
caudal	0 (0)^{a}	2 (2)^{ab}	0 (0)^{a}	0 (0)^{a}	4 (8)^{ab}	9 (33)^{b}	0 (0)^{a}	0 (0)^{a}
defects in ribs	4 (6)	0 (0)	3 (9)	1 (2)	5 (13)	6 (18)	4 (8)	3 (6)
				Visceral exam				
Number of litters examined internally (fetuses)	9 (48)	10 (57)	12 (44)	9 (40)	23 (141)	9 (57)	9 (46)	9 (45)
Cleft palate	1 (1)	2 (2)	0 (0)	0 (0)	2 (2)	2 (2)	0 (0)	0 (0)

Note. Data showing different superscripts (^{a,b,c}) indicate significant differences at $P < 0.05$.

ternal restraint did not enhance the developmental and neurobehavioral toxicity of arsenite at doses that were not teratogenic by themselves (8), and restraint stress on late gestation did not influence the effects of acute ethanol in pregnant mice (9). In the present study, although significant effects between caffeine and restraint stress were found in some maternal parameters, maternal restraint did not potentiate the effects of caffeine on fetal development.

In summary, although it has been demonstrated that both maternal restraint and caffeine administered at subteratogenic doses can enhance the teratogenic effects of a number of physical and chemical agents, no potentiative interactions between caffeine and restraint on embryotoxicity or teratogenicity were found. In contrast, there were some significant interactions (restraint × caffeine dose) on maternal parameters. However, taking into account that coffee may contain 75–150 mg caffeine per cup, to reach the highest dose of caffeine administered here (120 mg/kg), a 50-kg woman would have to drink 40 cups of strong coffee, which would be quite unusual. Notwithstanding, the known species differences in sensitivity must not be underestimated. Moreover, since the present results were based on a single exposure to caffeine in conjunction with 14 hr of restraint on Day 9 of gestation, further investigations should be carried out to assess whether other days of pregnancy or multiple days of maternal restraint stress/caffeine administration might have adverse effects on the fetuses.

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