Interactions of Caffeine and Restraint Stress During Pregnancy in Mice[1]

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The maternal and developmental toxicity of combined exposure to restraint stress and caffeine was assessed in mice. On gestational Days 0–18, three groups of plug-positive females (n = 13–15) were given by gavage caffeine at 30, 60, and 120 mg/kg/day. Three additional groups received the same caffeine doses and were restrained for 2 hr/day. Control groups included restrained and unrestrained plug-positive mice not exposed to caffeine. All animals in the group concurrently exposed to 120 mg/kg/day of caffeine and restraint died during the experimental period. In the remaining groups, cesarean sections were performed on Day 18 of gestation, and the fetuses were weighed and examined for external, internal, and skeletal malformations and variations. Although maternal and embryo/fetal toxicity were observed at all caffeine doses, the adverse maternal and developmental effects were significantly enhanced in the groups concurrently exposed to caffeine and restraint. It was especially remarkable at 60 and 120 mg/kg/day. The results of this study suggest that maternal and developmental toxic effects might occur if high amounts of caffeine were consumed by women under a notable stress during pregnancy. Exp Biol Med 227:779–785, 2002

Key words: mice; caffeine; maternal restraint; maternal toxicity; developmental toxicity; interactive effects.

Caffeine is a naturally occurring product that acts as a mild central nervous system stimulant. In humans, coffee, tea, and soft drinks, as well as cocoa, chocolate, and certain medications are the major sources of caffeine (1). Caffeine absorption from the gastrointestinal tract is rapid and reaches 99% approximately 45 min after ingestion. Due to the hydrophobic properties of the drug, it can cross the placenta and the brain-blood barriers. Caffeine half-life is lower in rodents than in humans and it is increased during pregnancy and the neonatal period (2).

Although caffeine passes readily through the placenta to the fetus (3), caffeine-containing products are also widely consumed during pregnancy. However, in mammals, caffeine is teratogenic only at extremely high doses that are also quite toxic to the dams, with the threshold dose for malformations appearing to be within the range of 80–100 mg/kg/day, depending on the method of administration and the species tested (4). Notwithstanding, reduced fetal body weight and delayed skeletal ossification have been observed at relatively high doses of caffeine (5). The developmental no-observed-effect-level (NOEL) in rodents seems to be approximately 30 mg/kg/day (4).

Results of epidemiological studies on potential reproductive and developmental toxicity of caffeine have not been consistent. In a recent investigation, Cnattingius *et al.* (6) corroborated earlier reports that found an increased risk of spontaneous abortion among pregnant women who ingested caffeine (7–9). These investigators concluded indicating that caffeine ingestion might increase the risk of an early spontaneous abortion among nonsmoking women carrying fetuses with normal karyotypes (6). In turn, Wen and co-workers (10) recently suggested that marked caffeine consumption during pregnancy might influence fetal viability in women with nausea, whereas Eskenazi *et al.* (11) reported that consumption of caffeinated coffee during pregnancy might be associated with a shortened gestation and a lowered birth weight, findings that confirmed previous investigations (12, 13). In contrast, other investigators did not find any relationship between spontaneous abortion and caffeine metabolites measured in blood (14). Moreover, in a recent review, Christian and Brent (4) pointed out that caffeine consumers were subjected to multiple confounding factors that made epidemiological analyses difficult.

On the other hand, in recent investigations performed in our laboratory, no interactive developmental toxicity in mice was found when caffeine (30, 60, and 120 mg/kg/day)

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and restraint stress (14 hr) were concurrently administered to pregnant mice on gestational Day 9 (15). A number of studies have demonstrated that maternal stress induced by noise, visual stimuli, heat, cold, or immobilization is capable of adversely affecting the developing conceptus (16–18). In addition, it has been also shown that maternal stress during pregnancy can enhance the adverse developmental effects of a number of chemicals (19–22). Recently, we found that prenatal stress in mice could slightly exacerbate the maternal and developmental toxicity of a single oral dose of caffeine (30 mg/kg) and aspirin (250 mg/kg) administered concurrently with 14 hr of restraint stress on gestational Day 9 (23). However, these results, as well as those of the previous study in which caffeine and restraint stress were concurrently administered to pregnant mice (15), were based on a single caffeine dose (gestation Day 9) in conjunction with 14 hr of restraint. During the entire gestational period, caffeine can be ingested by pregnant women who may also be potentially subjected to various types of stress in the home or workplace. Therefore, the present research was conducted to determine if a continued exposure to caffeine and stress during pregnancy might have adverse maternal and embryo/fetal effects.

Methods

Animals. Mature male and female Swiss Mice (Criffa, Barcelona, Spain) weighing 24–26 g were used. After a quarantine period of 7 days, female mice were mated with males (2:1) for 2 hr and were immediately examined for copulatory plugs. The day on which a vaginal plug was found was designated Day 0 of gestation. Animals were housed four per cage (polyethylene) and were kept in a climate-controlled animal facility at a temperature of 22° ± 2°C, a relative humidity of 50% ± 10%, and a 12:12-hr automatic light:dark cycle, with free access to food (Panlab rodent chow, Barcelona, Spain) and tap water. The use of animals and the experimental protocol were approved by the Animal Care and Use Committee of the "Rovira i Virgili" University.

Drug. Caffeine was purchased from Sigma Chemical Co. (St. Louis, MO). It was dissolved in deionized water and was administered by gavage at doses of 0, 30, 60, and 120 mg/kg/day during the entire gestational period (Days 0–18). Caffeine solutions were prepared daily and concentrations were adjusted to volumes of 0.20 ml/30 g body weight.

Treatment. On Day 0, plug-positive female mice were weighed and randomly assigned to one of eight groups (n = 13–15 animals per group). A control group was unrestrained and received deionized water by gavage. A second group of animals (restraint only control group) was immobilized for 2 hr/day on gestational Days 0–18 by placing the animals in metacrilate cylindrical holders from Letica Scientific Instruments (Panlab, Barcelona). 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 (15, 16, 19). Animals in the third, fourth, and fifth groups received caffeine only at doses of 30, 60, and 120 mg/kg/day on Days 0–18 of gestation, whereas mice in the sixth, seventh, and eighth groups were exposed to 30, 60, and 120 mg/kg/day of caffeine immediately followed by restraint stress for 2hr/day during the same days. All restrained animals were housed in the same room.

Data Collection. During the period of gestation, maternal body weight and food consumption were measured daily. On gestational Day 18, all animals were euthanized with diethyl ether, and the number of total implants, early and late resorptions, and live and dead fetuses were recorded. All live fetuses were dissected from the uterus and were evaluated for sex, body weight, and external malformations. To examine internal and skeletal abnormalities, they were randomly distributed in two groups. 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 (24). The remaining fetuses were fixed in Bouin's fluid, sectioned, and evaluated for internal abnormalities (25). All fetuses were examined by blind observers to the treatment conditions.

Data Analysis. The unit of comparison was the pregnant female or the litter. Results were analyzed by a two-way (restraint × caffeine dose) analysis of variance (ANOVA). *Post hoc* analyses were carried out with the Bonferroni method for multiple comparisons. Significance was set at the 0.05 probability level. The incidence of fetal anomalies between litters was compared by means of a two-tailed Fisher exact probability test for pairwise comparison of groups. In this case, significance was set at the 0.001 probability level.

Results

All mice in the group concurrently exposed to 120 mg/kg/day of caffeine and restraint stress died during the experimental period. Ten animals died on gestational Days 0×6, whereas the three remaining plug-positive females died on Days 7×15 of gestation. Consequently, no data on these animals are shown. In the remaining groups, only two dams died during the study at 60 mg/kg/day of caffeine only, and in the group concurrently exposed to caffeine at 30 mg/kg/day and restraint.

Maternal Toxicity. The effects of individual and/or concurrent exposure to caffeine and restraint on maternal body weight gain and food consumption are summarized in Table I. An overall effect of caffeine and restraint was observed on maternal body weight gain on gestational Days 0–18 [$F(3,73) = 5.45$, $P < 0.001$; $F(1,62) = 11.13$, $P < 0.001$]. Significant differences were observed between dams given 120 mg/kg/day of caffeine only and the control groups (restrained or unrestrained), as well as the group given caffeine only at 30 mg/kg/day. It can be seen that

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

Caffeine (mg/kg)	0	30	60	120	0	30	60
Type of stress	None	None	None	None	Restraint	Restraint	Restraint
Number of dams at term	13	10	14	10	11	10	13
Gestation days	Body weight gain (g)						
0–6	3.15 ± 0.99	3.00 ± 1.56	2.21 ± 1.85	1.90 ± 1.10	3.09 ± 1.04	1.60 ± 1.50	2.36 ± 1.01
7–15	15.77 ± 3.63^{a}	14.80 ± 2.14^{a}	12.28 ± 2.61^{ac}	8.20 ± 4.26^{bc}	14.00 ± 2.14^{a}	10.00 ± 3.33^{bc}	6.85 ± 4.79^{b}
16–18	9.92 ± 2.81^{a}	9.70 ± 2.31^{a}	8.43 ± 1.74^{ac}	5.00 ± 2.54^{b}	8.91 ± 2.07^{a}	6.40 ± 2.54^{bc}	3.77 ± 4.02^{b}
0–18	28.85 ± 6.67^{a}	27.50 ± 4.45^{ad}	22.93 ± 3.73^{ac}	15.10 ± 5.76^{bc}	26.00 ± 3.63^{ad}	18.00 ± 5.89^{bcd}	9.71 ± 14.15^{b}
	Food consumption (g)						
0–6	37.48 ± 9.39	48.25 ± 17.63	26.39 ± 2.99	28.47 ± 3.38^{a}	36.63 ± 4.58	39.71 ± 31.68	31.33 ± 3.84
7–15	77.95 ± 31.41^{a}	57.95 ± 14.08^{ab}	49.84 ± 3.60^{ab}	46.91 ± 3.60^{b}	65.36 ± 5.33^{ab}	63.96 ± 24.33^{ab}	49.43 ± 7.84^{ab}
16–18	15.11 ± 2.74^{ac}	31.87 ± 15.32^{b}	13.35 ± 3.21^{ac}	10.18 ± 2.86^{a}	16.29 ± 2.96^{ac}	20.88 ± 3.89^{cb}	10.41 ± 1.82^{a}
0–18	130.54 ± 37.21	138.08 ± 46.10	89.59 ± 5.72	85.56 ± 8.48	118.28 ± 10.90	127.76 ± 48.49	91.16 ± 11.96
	Mortality rate during gestation (%)						
	0	0	6.7	0	0	9.1	0

Note. Values are given as means ± SD. In each row, significant differences are indicated for comparisons between groups by the use of superscripts (a, b, c, and d). Data not showing a common superscript are significantly different at $P < 0.05$. Values showing a common superscript are not statistically significant ($P > 0.05$).

restraint stress significantly enhanced the adverse effect of caffeine on maternal weight gain at 60 mg/kg/day.

An overall effect of caffeine on food consumption was noted on gestational Days 0–6 [F(3,46) = 3.21, P = 0.033], 7–15 [F(3,46) = 6.04, P = 0.002], 16–18 [F(3,46) = 19.98, $P < 0.001$], and 0–18 [F(3,46) = 8.81, $P < 0.001$]. In turn, an overall effect of restraint was also observed on gestational Days 16–18 [F(1,46) = 4.37, P = 0.044]. However, significant differences among groups on food consumption were only found on gestational Days 7–15 and 16–18, when a decrease was observed in animals treated with the highest caffeine doses. In contrast, in the groups exposed to 30 mg/kg/day of caffeine, only a significant increase was noted on gestation Days 16–18.

Data on maternal variables recorded at gestational Day 18 are presented in Table II. A two-way (caffeine dose × restraint) ANOVA revealed an overall effect of caffeine and restraint [F(3,71) = 23.62, $P < 0.001$; F(1,71) = 32.42 $P <$ 0.001] on body weight at termination. Compared with the control group, body weight at termination was significantly decreased in mice treated with caffeine only at 120 mg/kg/day, as well as in dams concurrently exposed to 30 and 60 mg/kg/day of caffeine and restraint stress. An overall effect of caffeine was also detected on gravid uterine weight, corrected body weight (body weight at sacrifice minus gravid uterine weight), corrected body weight change (corrected body weight minus body weight on gestational Day 0), liver weight, and kidney weight [F(3,71) = 9.44, $P < 0.001$; F(3,71) = 16.89, $P < 0.001$; F(3,71) = 11.80, $P < 0.001$; F(3, 71) = 7.40, $P < 0.001$; F(3, 71) = 4.67, $P < 0.005$]. In turn, an overall effect of restraint was found on gravid uterine weight, corrected body weight, corrected body weight change, liver weight, and relative kidney weight [calculated as the percentage of corrected body weight; F(1,71) = 13.23, P = 0.001; F(1,71) = 6.22, P = 0.016; F(1,71) = 7.20, P = 0.010; F(1,71) = 7.27, P = 0.010]. However, no interactions between caffeine and restraint could be noted. Differences between groups in maternal variables were mainly observed in mice given caffeine at 60 mg/kg/day or in those concurrently exposed to caffeine and restraint.

Embryo/Fetal Toxicity. Data on pregnancy outcome measures are given in Table III. A two-way (caffeine dose × restraint) ANOVA revealed an overall effect of caffeine on the number of late resorptions and fetal body weight [F(3,71) = 3.09, P = 0.033; F(3,71) = 57.17, $P <$ 0.001], and an overall effect of restraint on the number of

Table II. Effects of Caffeine and Maternal Stress on Some Maternal Variables in Pregnant Mice

Caffeine (mg/kg)	0	30	60	120	0	30	60
Type of stress	None	None	None	None	Restraint	Restraint	Restraint
Number of dams	13	10	14	10	11	10	13
Body weight at termination (g)	54.85 ± 6.82^{a}	53.80 ± 4.80^{a}	49.14 ± 3.86^{ac}	40.20 ± 5.69^{b}	52.18 ± 4.28^{ac}	45.30 ± 5.06^{bc}	39.15 ± 8.6^{b}
Gravid uterine weight (g)	21.31 ± 3.90^{a}	20.59 ± 3.27^{ac}	16.68 ± 4.16^{ab}	14.55 ± 3.27^{bc}	19.97 ± 3.61^{ac}	13.73 ± 5.72^{b}	12.50 ± 5.04^{b}
Corrected body weight (g)	34.69 ± 2.76^{a}	33.21 ± 2.20^{ac}	32.45 ± 3.12^{ac}	28.07 ± 1.27^{b}	31.94 ± 1.9^{ac}	31.57 ± 1.77^{ac}	30.40 ± 2.3^{bc}
Corrected body weight change (g)	8.50 ± 1.90^{a}	6.91 ± 1.73^{a}	6.24 ± 2.94^{ac}	3.07 ± 1.57^{b}	5.94 ± 2.37^{ab}	4.27 ± 2.52^{b}	3.80 ± 1.24^{bc}
Liver weight (g)	2.24 ± 0.40	2.15 ± 0.23	2.03 ± 0.19	1.84 ± 0.23	2.15 ± 0.23	2.04 ± 0.31	1.95 ± 0.23
Relative liver weight (%)	6.55 ± 0.84	6.47 ± 0.48	6.29 ± 0.65	6.81 ± 0.48	6.73 ± 0.69	6.06 ± 1.73	6.41 ± 0.60
Kidney weight (g)	0.38 ± 0.04^{ab}	0.39 ± 0.04^{ab}	0.36 ± 0.03^{ab}	0.33 ± 0.03^{a}	0.40 ± 0.04^{b}	0.38 ± 0.06^{ab}	0.37 ± 0.05^{ab}
Relative kidney weight (%)	1.07 ± 0.10	1.16 ± 0.07	1.13 ± 0.10	1.16 ± 0.10	1.24 ± 0.15	1.30 ± 0.33	1.20 ± 0.10

Note. Values are given as means ± SD. In each row, significant differences are indicated for comparisons between groups by the use of superscripts (a, b, and c). Data not showing a common superscript are significantly different at $P < 0.05$. Values showing a common superscript are not statistically significant ($P > 0.05$).

Table III. Effects of Caffeine and Maternal Stress on Reproductive Findings

Caffeine (mg/kg)	0	30	60	120	0	30	60
Type of stress	None	None	None	None	Restraint	Restraint	Restraint
Number of litters	13	10	14	10	11	10	13
Implantation/litter	13.0 ± 3.2	14.0 ± 1.6	13.7 ± 2.4	12.0 ± 4.3	14.1 ± 3.1	12.5 ± 3.7	14.4 ± 3.1
Live fetuses/liter	11.8 ± 3.4^{ab}	13.2 ± 2.0^{b}	11.9 ± 2.3^{ab}	9.0 ± 5.3^{ab}	12.4 ± 3.0^{ab}	8.7 ± 4.4^{ab}	7.75 ± 5.5^{a}
Dead fetuses/litter	0.1 ± 0.3	0.2 ± 0.6	0.3 ± 0.6	0.0 ± 0.0	0.3 ± 0.5	0.3 ± 0.5	0.5 ± 1.0
Early resorptions/litter	0.9 ± 1.1	0.0 ± 0.0	0.8 ± 1.4	2.4 ± 3.1	0.9 ± 0.8	0.7 ± 1.6	3.6 ± 6.0
Late resorptions/litter	0.1 ± 0.4^{a}	0.6 ± 0.7^{ab}	0.7 ± 1.1^{ab}	0.6 ± 0.8^{ab}	0.5 ± 1.0^{ab}	2.8 ± 3.5^{b}	2.6 ± 3.1^{b}
Postimplantation loss litter (%)	8.67 ± 11.30^{a}	5.90 ± 6.06^{a}	12.58 ± 13.55^{a}	34.28 ± 36.6^{ab}	12.48 ± 7.28^{a}	30.34 ± 28.59^{ab}	46.00 ± 34.57^{b}
Mean fetal weight/litter (g)	1.38 ± 0.08^{a}	1.26 ± 0.14^{bd}	1.14 ± 0.9^{b}	0.92 ± 0.07^{c}	1.30 ± 0.11^{ad}	1.17 ± 0.14^{b}	0.97 ± 0.07^{c}
Fetal sex ratio (M/M + F)	0.5 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.3	0.5 ± 0.1

Note. Values are given as means ± SD. In each row, significant differences are indicated for comparisons between groups by the use of superscripts (a, b, c, and d). Data not showing a common superscript are significantly different at $P < 0.05$. Values showing a common superscript are not statistically significant ($P > 0.05$).

late resorptions and live fetuses, the percentage of postimplantation lost and fetal body weight [$F(3,71) = 11.98$, $P = 0.001$; $F(3,71) = 7.62$, $P = 0.007$; $F(3,71) = 15.82$, $P < 0.001$; $F(3,71) = 21.74$, $P < 0.001$]. As for maternal toxicity, no potentiative or synergistic effects between caffeine and restraint were found. However, the differences in the number of late resorptions, the percentage of postimplantation loss, and fetal body weight were significantly enhanced in the group concurrently exposed to caffeine at 60 mg/kg/day and restraint stress in comparison with the group given caffeine only at 60 mg/kg/day, which is indicative of an additive effect. Although fetal body weight was significantly reduced in all the caffeine-treated groups, the reductions were more notable in fetuses from dams concurrently exposed to caffeine and restraint. No differences between groups were observed in the number of total implantations, dead fetuses, and early resorptions.

On the other hand, no external, internal, or skeletal malformations were seen in fetuses of mice exposed to caffeine and/or restraint stress during pregnancy with the exception of cleft palate in the groups concurrently exposed to caffeine and restraint. However, some bone retardations could be noted in the experimental groups (Table IV). A two-way (caffeine dose × restraint) ANOVA revealed an overall effect of caffeine on the ossification of frontal, parietal, occipital, and jaw [$F(3,50) = 12.09$, $P < 0.001$; $F(3,50) = 14.44$, $P < 0.001$; $F(3,50) = 26.99$, $P < 0.001$; $F(3,50) = 6.07$, $P = 0.002$], sternebrae and xiphoid [$F(3,50) = 14.77$, $P < 0.001$; $F(3,50) = 24.98$, $P < 0.001$], metacarpus [$F(3,50) = 15.81$, $P < 0.001$), as well as the first and third anterior phalanges [$F(3,50) = 11.71$, $P < 0.001$; $F(3,50) = 9.27$, $P < 0.001$]. Metatarsus [$F(3,50) = 17.66$, $P < 0.001$], the first and the third posterior phalanges [$F(3,50) = 34.33$, $P < 0.001$; $F(3,50) = 11.68$ $P < 0.001$], and calcaneum and caudal nuclei [$F(3,50) = 4.60$, $P = 0.007$; $F(5,50) = 21.59$, $P < 0.001$] were also affected. However, neither significant effects of restraint nor interactions between caffeine and restraint could be observed.

A two-way (caffeine dose × restraint) ANOVA also revealed an overall effect of caffeine, and an interaction between caffeine and restraint, on cleft palate [$F(3,70) = 6.05$, $P < 0.001$; $F(2,70) = 3.63$, $P = 0.017$]. A significant increase in the number of litters with fetuses affected with cleft palate was noted in the group concurrently exposed to 60 mg/kg/day of caffeine and restraint (Table IV).

Discussion

The results of the current study show that caffeine administration to pregnant mice on gestational Days 0–18 at doses of 120 mg/kg/day has adverse maternal effects that are evidenced by a significant reduction in body weight gain on Days 7–15, 16–18, and the entire gestational period (Days 0–18). The reduction on gestation Days 0–18 was independent on food consumption, as no significant differences between groups could be seen in this parameter. Moreover, in this group, body weight at termination, gravid uterine weight, corrected body weight, and corrected body weight change were also significantly decreased, not only in relation to the control group, but also in comparison with the groups given caffeine at 30 and 60 mg/kg/day (with the exception of gravid uterine weight). However, no significant differences between the groups exposed to 30 and 60 mg/kg/day of caffeine could be noted. With regard to caffeine-induced developmental toxicity, adverse effects were observed at 60 and 120 mg/kg/day. These effects were significantly more notable at 120 than at 60 mg/kg/day.

In a previous study, Elmazar *et al.* (26) reported retarded ossification, particularly of the supraoccipital bone, in fetuses of mice when caffeine (150 mg/kg/day) was given in the drinking water from Days 5–18 of pregnancy, but not when the same dose was given as a sustained release pellet. The current results are in agreement with the conclusions of a review on the potential teratogenic of coffee and caffeine exposure in which it was shown that the embryo/fetal toxicity of caffeine in mice appeared at doses of 50–75 mg/kg/day (27). In contrast, the results of the present study are quite different from those obtained in a recent investigation in which caffeine was given in a single dose on gestational Day 9. No adverse effects of caffeine on dams and offspring were then noted (15).

The most striking finding of the current investigation is the interaction of restraint stress and caffeine on the maternal and developmental toxic effects. Although in the group subjected to restraint only, no adverse effects were observed, the maternal and developmental toxicity of caffeine was significantly enhanced when the dams were concur-

Table IV. Effects of Caffeine and Maternal Stress on Morphological Defects in Mouse Fetuses

Caffeine (mg/kg)	0	30	60	120	0	30	60
Type of stress	None	None	None	None	Restraint	Restraint	Restraint
				Skeletal examination			
Number of litters examined skeletally (fetuses)	13 (78)	10 (68)	14 (86)	8 (46)	11 (75)	10 (45)	9 (49)
Reduced ossification							
Frontal	0 (0)^{a}	3 (8)^{ab}	6 (21)^{ab}	4 (16)^{ab}	0 (0)^{a}	3 (5)^{ab}	8 (35)^{b}
Parietal	0 (0)^{a}	3 (8)^{a}	5 (10)^{a}	8 (33)^{b}	1 (3)^{a}	1 (2)^{a}	7 (30)^{ab}
Occipital	0 (0)^{a}	3 (5)^{ac}	8 (26)^{b}	7 (40)^{b}	1 (1)a	2 (3)^{a}	8 (36)^{bc}
Jaw	0 (0)^{a}	0 (0)^{ab}	1 (3)^{ab}	4 (6)^{b}	0 (0)^{ab}	1 (2)^{ab}	3 (8)^{ab}
Sternum	2 (2)^{a}	4 (7)^{ab}	10 (33)^{bc}	3 (13)^{ab}	3 (5)^{a}	4 (5)^{ab}	9 (39)^{b}
Xiphoid bipartite	0 (0)^{a}	1 (1)^{a}	6 (15)^{ab}	3 (16)^{b}	0 (0)^{a}	1 (1)^{ac}	8 (28)^{b}
Caudal vertebrae	0 (0)^{a}	4 (14)^{ab}	9 (60)^{bc}	8 (46)^{b}	2 (11)^{ac}	4 (6)^{c}	8 (42)^{b}
Proximal anterior phalanges	2 (2)^{a}	1 (1)^{a}	7 (20)^{ab}	8 (44)^{b}	1 (1)^{a}	1 (2)^{a}	7 (32)^{b}
Middle anterior phalanges	6 (15)	7 (31)	12 (67)	8 (46)	10 (51)	7 (23)	9 (49)
Distal anterior phalanges	2 (3)^{a}	1 (1)^{a}	8 (26)^{ac}	8 (44)^{b}	3 (4)^{ac}	1 (2)^{a}	8 (21)^{bc}
Metacarpal	0 (0)	0 (0)	1 (1)	2 (6)	0 (0)	0 (0)	3 (7)
Proximal posterior phalanges	0 (0)^{a}	3 (3)^{ac}	10 (29)^{bc}	8 (46)^{b}	2 (3)^{a}	3 (5)^{ac}	8 (38)^{b}
Middle posterior phalanges	10 (45)	10 (68)	14 (86)	8 (46)	11 (70)	10 (44)	9 (49)
Distal posterior phalanges	1 (1)^{a}	2 (2)^{a}	8 (20)^{ab}	8 (45)^{b}	2 (3)^{a}	3 (5)^{ab}	8 (34)^{b}
Metatarsal	0 (0)	0 (0)	1 (1)	2 (6)	0 (0)	0 (0)	2 (4)
Calcaneous	6 (13)^{a}	9 (47)^{b}	14 (67)^{b}	8 (46)^{b}	10 (35)^{b}	10 (30)^{b}	9 (49)^{b}
Supernumerary ribs	3 (5)	5 (11)	3 (4)	1 (1)	3 (5)	4 (6)	2 (5)
Total skeletal variations	10 (46)	10 (68)	14 (86)	8 (46)	11 (70)	10 (44)	9 (49)
				Internal examination			
Number of litters examined internally (fetuses)	13 (75)	10 (64)	14 (80)	8 (43)	11 (62)	10 (42)	9 (57)
Cleft Palate	0 (0)^{a}	1 (1)^{a}	1 (1)^{a}	1 (1)^{a}	0 (0)^{a}	3 (6)^{a}	6 (7)^{b}
				Skeletal and internal examination			
Total number of litters examined (fetuses)	13 (153)	10 (132)	14 (166)	8 (89)	11 (137)	10 (87)	9 (106)
Total number of internal and skeletal malformations and variations (fetuses)	10 (46)	10 (69)	14 (87)	8 (47)	11 (70)	10 (50)	9 (56)

Note. In each row, significant differences are indicated for comparisons between groups by the use of superscripts (a, b, and c). Data not showing a common superscript are significantly different at $P < 0.001$. Values showing a common superscript are not statistically significant ($P > 0.001$).

rently subjected to restraint. It was especially noteworthy in the group given 120 mg/kg/day caffeine, in which all dams died during the study. In turn, dams in the group concurrently exposed to 60 mg/kg/day of caffeine and restraint showed a significant reduction in body weight gain and body weight at termination in comparison with mice given 60 mg/kg/day of caffeine only. Although corrected body weight change was also lower in the groups exposed to caffeine (60 mg/kg/day) and restraint, the differences between both groups did not reach the level of statistical significance ($P > 0.05$). Significant differences in the number of live fetuses per litter, percentage of postimplantation loss, fetal body weight, and the number of litters with reduced ossification in a number of bones were also observed between these groups, with restraint significantly enhancing the embryo/fetal toxicity of caffeine. In addition, significant differences between the group exposed to 30 mg/kg/day of caffeine only and the group concurrently exposed to this dose of caffeine and restraint were also found in maternal weight gain on gestational Days 7–15 and 16–18, and body weight at termination, gravid uterine weight, and corrected body weight change. All these maternal variables were lower in the group concurrently exposed to caffeine and restraint stress. Although the data seem to point out that an adverse influence of restraint could have occurred, most differences between the restrained and unrestrained caffeine (30 mg/kg/day)-treated groups were not statistically significant.

The present results clearly indicate the existence of an additive interaction between caffeine and restraint stress that significantly enhances the maternal and developmental toxicity of caffeine in mice. Although the current findings are new, the presence of interactive effects in developmental toxicity induced by concurrent exposures has not been especially surprising (28–30). In a wide and interesting review on interactions in developmental toxicology (30), of approximately 160 reports of concurrent exposure reviewed, about one-third reported potentiative or synergistic effects. In relation to caffeine, it has been demonstrated that this drug potentiates the teratogenic effects of nicotine, ethanol, some alkylating agents, and irradiation (31–35). Moreover, in a recent study in our laboratory, the incidence of some skeletal defects in mouse fetuses was significantly increased in the binary and ternary combinations of caffeine (30 mg/kg), aspirin (250 mg/kg), and restraint stress given at single doses on gestational Day 9 (23).

On the other hand, in recent years, a number of studies have shown that maternal stress might have a notable influence on the adverse maternal and embryo/fetal effects of some developmental toxicants. Interactive effects in developmental toxicity have been reported to occur in pregnant animals exposed to salicylate and restraint (36), ethanol or arsenic and hyperthermia (37, 38), all-trans-retinoic acid and restraint (39, 40), and some metals (aluminum, arsenic, and methyl mercury) and restraint (16, 20–22, 41).

Nehlig and Debry (27) reported that the daily consumption of caffeine in the general population ranges from 203 to 283 mg, which means 2.7–4.0 mg/kg/d of caffeine in adult subjects. In 1980, the U.S. FDA recommended that pregnant women limit caffeine consumption to less than 400 mg/day (6.7 mg/kg/day for 60-kg body weight) on the basis primarily of results of animal studies (42). According to the results of the current study and taking into account the potential additive effects of caffeine and maternal stress, the consumption of 30 mg/kg/day of this drug could mean a certain hazard in stressed (stressful jobs, style of life, etc) pregnant women. Usually, the greatest amount of caffeine is ingested from coffee, which may contain between 75 and 150 mg of the drug per cup. Consequently, to reach a daily ingestion of 30 mg/kg of caffeine, a 60-kg woman would have to drink 12 cups of a very strong coffee, a quantity that is very unusual. However, the present results show that if 30 mg/kg/day of caffeine are concurrently administered with maternal stress, this dose is then a lowest observable adverse effect level instead of a no-observable adverse effect level. Moreover, it has been reported that stress can also result in increased use of substances such as caffeine, tobacco, or alcohol (43).

In summary, although few epidemiological data have connected caffeine consumption with preterm delivery or congenital malformations, reproductive adverse effects of caffeine such as infertility, spontaneous abortion, or low birth weight cannot be discarded (6, 7, 10, 11, 44, 45). Consequently, and in spite of interspecies differences in the bioavailability and pharmacokinetics of caffeine, as a preventive point of view, we suggest that those women under a notable stress during pregnancy significantly reduce caffeine ingestion to reasonable levels. For example, a dose of 10 mg/kg/day of caffeine means, for a 60-kg woman, a daily ingestion of 600 mg; that is to say, four cups of a strong coffee or eight cups of a weak coffee.

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