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Effects of Caffeine on the Growth of Mandible and Long Bone in Protein–Energy Malnourished Newborn Rats (41911)

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Abstract. Rat dams with eight pups each were divided into six groups upon delivery; the first three were fed 6, 12 or 20% protein diets, and the second three the same diets but with caffeine added in the amount of 2 mg/100 g body wt. At Day 15, randomly selected pups were injected with [^{14}C]proline to determine collagen synthesis of mandible and long bone. Other pups were used to determine the calcium content of these bones. The body, mandibular, and long bone weight of the pups whose dams were fed the 6% protein diet with caffeine increased compared to the noncaffeine group. Calcium content of the mandible and the collagen synthesis of the long bone were also increased. However, calcium content of long bone, collagen synthesis of mandible, and hydroxyproline content of mandible and long bone showed no difference between the caffeine and noncaffeine groups. In the pups whose dams were fed the 12% protein diet with caffeine, body and mandibular weight, collagen synthesis, and hydroxyproline and calcium contents in mandibles and long bones of pups showed no difference from those of the noncaffeine group, but long bones were heavier. In the pups whose dams were fed the 20% protein diet with caffeine, the body and long bone weight and hydroxyproline and calcium contents of the long bone of pups were lower than those of the noncaffeine group. Mandibular weight, calcium content, and hydroxyproline showed no difference between caffeine and noncaffeine animals, but collagen synthesis of the mandible was increased. Current data indicate that nutritional state and caffeine intake of the mother have a close relation to growth and development of the offspring.

Caffeine is found in coffee, tea, cola, and other carbonated soft drinks as well as in many popular, over-the-counter medications such as cold and allergy tablets, headache tablets, diuretics, and stimulants (1). Recently, the number of women who nurse their infants has shown a gradual but steady increase. By 1975, 52% of women with more than 12 years of schooling were breast-feeding their infants (2). Since caffeine is known to diffuse easily into breast milk (3) and the elimination half-life of caffeine in infants is much longer than in adults (4), a growing breast-fed infant may easily be affected by the mother's caffeine intake. The interaction of the mother's nutritional state and caffeine intake on infant bone has been paid scant attention. This is important, because socioeconomic factors may lead to various nutritional deficiencies in mothers as well as their infants.

It has been reported that some nutritional conditions may not only modify the toxicity of chemical products and the therapeutic efficacy of some drugs (5), but also may modulate tissue sensitivity to drugs (6). In the present study, two bones, the mandible, an example of the membranous bone, and a long bone, an example of the endochondral (7), were selected to study how the interaction of the mother's nutritional state and caffeine intake alter offspring bone growth and development during the early postnatal period.

Materials and Methods. Twenty-six pregnant Sprague–Dawley rats (Holtzman strain) were fed a standard laboratory chow until the pups were delivered. Litters delivered within 8 hr of each other were combined. Because the sex of newborn rats does not account for any significant physiological differences (8), each dam received eight pups picked randomly, and the pups' birth dates were designated as Day 1. The first group of dams (group A; $n = 14$) was divided randomly into three subgroups and fed a 6% ($n = 4$), 12% ($n = 5$), or 20% ($n = 5$) protein diet, derived from casein. The second group of dams (group B; $n = 12$) was also divided randomly into three subgroups ($n = 4$ for each group) and pair-fed with the group A rats. The diets were identical except for the

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TABLE I. DIETARY COMPOSITION (g)

	6%	6% + Caffeine	12%	12% + Caffeine	20%	20% + Caffeine
Casein	60	60	120	120	200	200
Dextrose	263	263	231	231	192	192
Sucrose	180	180	180	180	178	178
Dextrin	258	258	230	230	192	192
Mazola corn oil	150 ml	150 ml	150 ml	150 ml	150 ml	150 ml
Mineral mix[a]	40	40	40	40	40	40
Choline chloride, 50% (w/v)	4 ml	4 ml	4 ml	4 ml	4 ml	4 ml
Cellulose	35	35	35	35	35	35
Vitamin mix[b]	10	10	10	10	10	10
Methionine	1	1	1	1	—	—
Caffeine	—	0.29	—	0.23	—	0.20

[a] Roger–Harper Mineral Mix (Teklad Test Diets, Madison, Wisc.).
[b] AIN Vitamin Mixture 76 (ICN Pharmaceuticals, Inc., Cleveland, Ohio).

addition of caffeine to the group B diet, in amounts calculated to provide daily doses of 2 mg/100 g body wt.[1] Their diets were isoenergetic with the 20% protein groups as a control, the difference in energy being made up by the addition of dextrose and dextrin (8) (Table I).

The dams and pups were weighed every other day. At Day 15, randomly selected pups from each dam in each group received an injection of uniformly labeled [^{14}C]proline (New England Nuclear, Boston, Mass.) at a dose of 6.5 μCi/100 g ip and immediately returned to their dam. Six hours later, the treated pups were sacrificed by severing the carotid artery, and the blood was collected, in order to determine the precursor pool of [^{14}C]proline (9).

The left and right mandibles of each pup were separated by splitting in the middle with a sharp knife. The tooth germs of molars and incisors, and the soft tissue and mandibular nerves from the mandibular body were removed. The distal part of the femur, the knee joint, and the proximal part of the tibia were similarly removed and cleaned. Specimen cleanliness was verified under a 10× hand-magnifying glass (8).

The specimens were weighed and dried overnight at 110°C. Three mandibles or long bones selected at random were placed in 10-ml ampoules containing 1.5 ml of 6 *N* HCl. The ampoules were flushed with nitrogen for 30 sec, sealed immediately, and placed in an oven at 125°C for 24 to 30 hr. After cooling, the hydrolysates were filtered through a fritted disc funnel using a vacuum pump, and a 1-ml aliquot was diluted with 3 ml of sodium acetate–citrate buffer, pH 6. The addition of 6 ml of 5% Na_2CO_3 neutralized the acid (9). Total proline (10), total hydroxyproline (11), and the radioactivity of [^{14}C]proline and [^{14}C]hydroxyproline were then measured as described previously (9, 12).

Mandibles and long bones of the remaining pups from each dam in each group were removed and prepared to determine Ca content. A cleaned mandible or long bone was oxidized with 0.2 ml of 60% perchloric acid and 0.4 ml of 30% H_2O_2 in a 75°C oven for 1 hr (13). Calcium content of the oxidized samples was determined by atomic absorption spectrophotometry (Model 280, Fisher Scientific Co., Fair Lawn, N.J.). Data were analyzed using Student's *t* test.

Results. The body weight of pups from the 6% protein with caffeine group was greater than that of the noncaffeine group at Days 11, 13, and 15. The statistical significance increased as the experiment progressed ($P < 0.05$ to $P < 0.01$). The body weight of the pups from the 12% protein with caffeine group showed no difference throughout the

[1] Based on the average body weight and the mean daily food intake in the dams fed each of the different casein diets during Days 1 to 15 of the postnatal period; the amount of caffeine was adjusted to 2 mg/100 g body wt. Daily food intake means (g/day) for the dams fed the protein diets either with or without caffeine were 30.9 ± 8.6 vs 33.9 ± 6.5 in the 20% protein group, 25.2 ± 4.3 vs 26.2 ± 3.5 in the 12% protein group, and 15.9 ± 2.3 vs 16.4 ± 1.8 in the 6% protein group.

experimental period when compared to the noncaffeine group. The body weight of the pups from the 20% protein with caffeine group was less at Days 7, 9, 11, 13, and 15 than that of the noncaffeine group ($P < 0.001$) (Table II).

The mandibular weight of the pups from the 6% protein with caffeine group was more than that of the pups from noncaffeine dams ($P < 0.05$). However, for those on the 12 or 20% protein diet caffeine made no difference ($P > 0.05$). The long bones of the pups from the 6 and 12% protein with caffeine groups were heavier than those without caffeine ($P < 0.01$). In contrast, the long bone weights of the pups from the 20% protein plus caffeine group were less than those from the noncaffeine animals ($P < 0.001$) (Table III).

Adding caffeine to the 6, 12, and 20% protein diets showed no effects in hydroxyproline content per mandible or gram of tissue in the newborn ($P > 0.05$). In contrast, hydroxyproline content of the long bones from pups of the 20% protein with caffeine group was less than the hydroxyproline content of the noncaffeine pups on the 20% protein diet ($P < 0.05$). When the data were expressed per gram of tissue, there was no difference. No differences in hydroxyproline content in the long bones from either 6 or 12% protein diets were evident between caffeine-supplemented and noncaffeine groups ($P > 0.05$) (Table III).

Collagen synthesis in the mandibles from the offspring of the 20% protein diet group supplemented with caffeine was increased over that in the comparable noncaffeine group ($P < 0.05$), but no such effects were evident between caffeine-supplemented and noncaffeine groups on the 6 or 12% protein diets ($P > 0.05$). Collagen synthesis of the long bone in individuals whose dams were supplemented with caffeine in the 6% protein diet increased compared to their noncaffeine counterparts ($P < 0.01$). However, there was no difference between caffeine-supplemented and noncaffeine animals in the 12 and 20% protein diet groups ($P > 0.05$) (Table III).

Calcium content per mandible or gram of tissue in the 6% protein with caffeine pups was higher than that in the noncaffeine group ($P < 0.001$). However, there was no such difference between caffeine and noncaffeine groups on the 12 and 20% protein diets. While the calcium content per long bone or gram of tissue of the caffeine-supplemented group on the 20% protein diet was less than that of the noncaffeine group ($P < 0.001$), no such difference appeared between the caffeine and noncaffeine groups nourished by dams fed the 6 or 12% protein diets (Table III).

Discussion. That caffeine consumed by dams can pass through their milk to their offspring is well known (14). Whereas the toxic effects of high doses of caffeine injected (10–17.5 mg/100 g body wt) into newborns have been observed as poor weight gain and/or high mortality (15), the effect of caffeine through lactating dams on suckling pups has been paid little attention. Since caffeine intake by pups during lactation is estimated to be about 2% of that received by their nursing mothers (14), those in the present study received much smaller dosages of caffeine than previous studies (15).

An unexpected finding was the increase in body weight of the 6% protein plus caffeine pups. Although the exact mechanism involved here has to be elucidated in future studies, some possible explanations are suggested. Slower degradation of caffeine in infants (4) may have been prolonged further by the decreased intake of protein. It has been shown that a low protein diet in general further increased the half-life of drugs (16, 17). Malnutrition's exaggerated tissue sensitivity to other drugs has been documented (6, 18), so tissue sensitivity to caffeine may have enhanced protein synthesis (19) in various organs, as was observed in brain tissue (20). It could also be possible that the caffeine exerted some type of stimulating effect, such as feeding longer or more vigorously, on pups suckled by dams fed the 6% protein diet. In the present study, consistently higher, though statistically not significant, values of collagen contents in both mandible and long bone in the 6% protein with caffeine group were observed compared to that of the noncaffeine group.

On the other hand, as protein in the caffeine-supplemented groups was increased to 12 and 20%, the beneficial weight gain effect observed in the 6% protein plus caffeine group did not appear. Some apparently deleterious growth effects did occur in the 20% protein with caffeine animals, viz., lower

TABLE II. CHANGES IN BODY WEIGHT (g)

		Day							
		1	3	5	7	9	11	13	15
6% protein	No caffeine	6.64 ± 0.05[a] (30)[b]	9.34 ± 0.17 (32)	9.68 ± 0.25 (20)	11.90 ± 0.27 (24)	12.96 ± 0.34 (24)	13.65 ± 0.26 (32)	14.76 ± 0.32 (24)	15.17 ± 0.36 (32)
	Caffeine		9.33 ± 0.15 (32)	10.02 ± 0.16 (25)	12.39 ± 0.24 (24)	13.27 ± 0.24 (24)	14.81 ± 0.28[c,*] (16)	15.75 ± 0.24* (32)	16.64 ± 0.26** (32)
12% protein	No caffeine	6.58 ± 0.06 (30)	9.89 ± 0.10 (40)	12.41 ± 0.22 (22)	14.46 ± 0.21 (30)	18.06 ± 0.27 (16)	19.23 ± 0.59 (24)	22.82 ± 0.40 (24)	23.04 ± 0.56 (40)
	Caffeine		8.98 ± 0.14 (32)	11.67 ± 0.21 (20)	14.85 ± 0.26 (32)	17.42 ± 0.23 (25)	20.14 ± 0.29 (32)	22.51 ± 0.28 (32)	24.38 ± 0.32 (32)
20% protein	No caffeine	6.60 ± 0.05 (30)	9.53 ± 0.12 (40)	13.82 ± 0.15 (40)	18.52 ± 0.15 (40)	23.13 ± 0.17 (40)	28.00 ± 0.26 (40)	32.53 ± 0.27 (40)	36.95 ± 0.26 (40)
	Caffeine		9.43 ± 0.13 (32)	13.30 ± 0.25 (24)	16.87 ± 0.25** (32)	20.86 ± 0.31*** (32)	25.54 ± 0.53*** (24)	29.41 ± 0.60*** (32)	33.05 ± 0.69*** (32)

[a] The values represent means ± SEM.
[b] The number of animals randomly weighed is given in parentheses.
[c] Significantly different from noncaffeine group of the same age in respective protein diet group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

TABLE III. VARIOUS PARAMETERS STUDIED ON A MANDIBLE AND LONG BONE AT DAY 15

		Weight (mg)		Hydroxyproline (μmole)		RSA[b]		Calcium (mg)	
		M[a]	LB[a]	M	LB	M	LB	M	LB
6% protein	No caffeine	44.34 ± 0.56[c] (12)[d]	55.97 ± 1.24 (12)	3.10 ± 0.15 (6)	3.35 ± 0.17 (6)	0.211 ± 0.011 (6)	0.141 ± 0.002 (6)	3.31 ± 0.08 (9)	1.63 ± 0.06 (9)
	Caffeine	47.17 ± 0.91[e,*] (12)	61.03 ± 1.14* (12)	3.47 ± 0.14 (6)	3.73 ± 0.17 (6)	0.245 ± 0.012 (6)	0.166 ± 0.006** (6)	4.03 ± 0.08*** (8)	1.71 ± 0.11 (8)
12% protein	No caffeine	61.45 ± 2.64 (10)	79.32 ± 2.36 (12)	4.40 ± 0.24 (6)	4.70 ± 0.18 (6)	0.231 ± 0.005 (6)	0.191 ± 0.005 (6)	4.79 ± 0.33 (9)	2.42 ± 0.22 (9)
	Caffeine	62.73 ± 0.70 (12)	89.20 ± 2.34** (12)	4.60 ± 0.13 (6)	4.85 ± 0.18 (6)	0.235 ± 0.009 (6)	0.189 ± 0.007 (6)	4.91 ± 0.08 (8)	2.53 ± 0.15 (8)
20% protein	No caffeine	73.47 ± 0.59 (12)	126.40 ± 1.42 (12)	5.25 ± 0.28 (6)	6.37 ± 0.16 (6)	0.291 ± 0.008 (6)	0.232 ± 0.009 (6)	5.83 ± 0.29 (10)	4.53 ± 0.08 (10)
	Caffeine	69.15 ± 1.84 (11)	112.71 ± 2.64*** (10)	4.73 ± 0.18 (6)	5.87 ± 0.13* (6)	0.348 ± 0.009* (6)	0.226 ± 0.006 (6)	5.64 ± 0.19 (8)	3.56 ± 0.21*** (8)

[a] M = mandible, LB = long bone.
[b] RSA = relative specific activity. RSA (bone hydroxyproline sp act/blood proline sp act) was calculated to provide an index of the uptake and/or metabolic conversion of proline by the tissue under consideration relative to that in the precursor pool as described previously (9).
[c] The values represent means ± SEM.
[d] Number of samples determined is given in parentheses.
[e] Significantly different from noncaffeine group in respective protein diet group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

body weight, lower long bone weight, lower hydroxyproline content, and lower long bone calcium content.

In the present study, caffeine was adjusted to the dam's weight and food intake. Theoretically, regardless of the differences in food intake, dams received a constant dose of caffeine per gram of body weight. Assuming that caffeine excretion into milk is constant per unit volume milk, regardless of the nutritional status of the dams, then pups in the 6% protein diet group with caffeine would have received less caffeine than the pups of the 12 and 20% protein with caffeine group, since milk in the 6% protein diet group was less than that of the 12 or 20%-protein diet (21).

The body weight of the pups increased proportionately as maternal dietary protein content increased. Maternal protein in the diet plays a critical role in the growth and development of the offspring (21, 22). It is conceivable that higher milk volume accompanied by greater caffeine passage to offspring in the 20% protein group may have partially caused the decreased body weight when compared to the noncaffeine groups. This view may be supported by the fact that the body weights were about the same among individuals from the 12% protein diet with and without caffeine and suggests that at this protein level, caffeine in newborns has no effect on body growth.

Low and high carbohydrate contents in the 20 and 6% protein diets, respectively, may raise the question of whether the presently observed effects of caffeine on pups may partly be due to the result of some interaction with the carbohydrate components of the maternal diets. Although the possibility of the interaction existing with the carbohydrate which is about 70% by weight in the 6% protein diet, and 56% by weight in the 20% protein diet, a difference of 14%, cannot be ruled out, the increase of the protein content from 6 to 20% in the diet accounts for an increase over 300%. It is, thus, likely that the protein portion of the diet would have had a greater effect than that of the carbohydrate portion on fetal growth.

It has been reported that because the limiting nutrient in rat milk is protein, the offspring experience a kind of protein–energy malnutrition when a protein-deficient diet is fed to the dams (8, 21). While the quality of milk is not changed as a result of protein or energy restriction, its quantity is decreased (21).

In general, trends in the weight changes in both mandible and long bone in caffeine and noncaffeine animals resembled that of the body weight changes. The ratio of organ weight to total body weight in each group, in fact, was practically the same in both the caffeine and noncaffeine pups. Supplementation with caffeine in the 6% protein diet significantly increased the weight of body mandible and long bone. The effects of caffeine, however, on these bones differed slightly in the 12% and 20% protein groups.

In the present *in vivo* study, collagen synthesis of the mandible and long bone in the offspring whose dams were fed with caffeine differed somewhat from those of noncaffeine animals. This suggests that supplementation of caffeine and the nutritional state of the newborn interact differently on the mandible and long bone.

It has been reported that the total hydroxyproline content of bones reflects their collagen content (23). When the rate of catabolism is reduced below the rate of synthesis, the total amount of collagen will increase (24). Interrelationships between catabolism and synthesis on the collagen contents of the tissue have been described (25). In the present study, collagen synthesis and collagen content of the individuals that received caffeine with various levels of maternal protein were different from comparable noncaffeine groups, suggesting again the interaction of the nutritional status of the offspring and their caffeine intake.

Calcium content of the mandible and long bone of the offspring also differed in caffeine and noncaffeine pups among different maternal protein groups. It is probable that the influence of the dietary protein source on the bone cell metabolism may be an important factor. This effect would be in keeping with published reports that high dietary protein during growth affects calcium absorption and thereby calcification (26); others (27), however, have shown that the protein content of the diet does not influence calcium absorption.

The increase or decrease of the weight of mandible and long bone in diets supple-

mented with caffeine compared with those of noncaffeine animals in the groups may partly be explained on the basis of calcium and collagen contents of the respective bones. Although the values for collagen and calcium in the long bones of the 6 and 12% groups with caffeine did not achieve statistical significance over the noncaffeine groups, their values were consistently higher. This trend is in agreement with the increased weight of the long bones themselves. The present investigation demonstrates that caffeine taken by lactating dams (2 mg/100 g) can affect their nurslings in the various parameters studied.

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