Prenatal effects of maternal caffeine intake and dietary high protein on mandibular development in fetal rats

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The purpose of the present study was to determine the effects of caffeine on the mandibles of newborn rats whose dams were given a normal diet (200 g protein/kg diet) compared with those given a high-protein diet (400 g protein/kg diet) during gestation. A total of twenty pregnant Sprague-Dawley rats were randomly divided into four groups of five each. Starting on day 7 of gestation, groups 1 and 2 were fed on control and high-protein diets respectively, and groups 3 and 4 were pair-fed with groups 1 and 2 respectively, but with caffeine added to their diets. The caffeine supplement was 20 mg/kg body-weight. At birth, pups were killed and various measurements of their mandibles were made. The mandibular weights, calcium contents, and alkaline (*EC* 3.1.3.1) and acid (*EC* 3.1.3.2) phosphatase activities of the group given the caffeine-supplemented control diet were significantly lower than those of the corresponding unsupplemented group. Alkaline and acid phosphatase activities, collagen synthesis and hydroxyproline contents of the caffeine-supplemented high-protein group were greater than those of the corresponding unsupplemented group, whereas Ca and protein contents of the caffeine-supplemented high-protein group were lower than those of the corresponding unsupplemented group. There were no significant differences in plasma caffeine levels for either dams or pups between the caffeine-supplemented control and high-protein groups. The effects of caffeine on the development of fetal mandibles are apparently modified by different levels of maternal dietary protein.

Caffeine: Mandibular development: Dietary protein: Rat

Caffeine is present in many everyday household items which are widely used in our society. For example, it is present in coffee, tea and many carbonated drinks, and over-the-counter drugs used for allergies and colds (Graham, 1978). Caffeine has been shown to produce palatal and limb defects in fetal rats and to have an effect on chondrogenesis in the limbs (Nishimura & Nakai, 1960). It may also cause the release of catecholamines from maternal adrenal glands and fetal tissue. Catecholamines cause a disturbance in circulation and decrease blood supply to the uterine vessels which may affect the growing fetuses (Moriguchi & Scott, 1986).

In spite of the concern about caffeine, the majority of women still consume coffee and various other caffeine-containing products during pregnancy (Graham, 1978). Women who drink more than five cups of coffee daily are more likely to have premature labour, a small fetus for the gestational period and a high incidence of an impending abortion (Furuhashi *et al.* 1985).

Diets in the US are reported to be relatively high in protein. The amount of protein consumed by pregnant women surveyed was 14–43 % above the recommended daily allowance (Chopra *et al.* 1978). An excess of dietary protein with a moderate intake of calcium results in a hypercalciuria in humans (Kim & Linksweiler, 1979). In animal studies, excess dietary protein with adequate Ca has shown no effects on bone metabolism in

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Table 1. *Composition of diets (/kg)*

Protein level (g/kg) ...	200	400
Casein (g)	200	400
Glucose (g)	192	122
Sucrose (g)	178	118
Dextrin (g)	192	122
Maize oil (Mazola) (ml)	150	150
Mineral mix* (g)	40	40
Choline chloride (500 g/l) (ml)	4	4
Cellulose (g)	35	35
Vitamin mix† (g)	10	10
Caffeine	‡	‡

* Rogers–Harper Mineral Mix (Teklad Test Diets, Madison, WI).
† AIN Vitamin Mixture 76 (ICN Pharmaceuticals Inc., Cleveland, OH).
‡ 20 mg/kg body-weight.

growing rats (Allen & Hall, 1978) or adult mice (Yuen & Draper, 1983). However, it is not clear at the present time to what extent, if any, the maternal high-protein diet with adequate Ca intake during the gestational period can influence bone development of the growing fetuses.

The present studies were conducted to determine whether or not the interaction between maternal high protein intake and caffeine supplementation would affect the development of the fetal bones. In addition, using high-performance liquid chromatography (HPLC), plasma caffeine levels in the dams and their pups were measured to investigate whether these levels might be altered by the nutritional status of the rats.

MATERIALS AND METHODS

A total of twenty timed-pregnant Sprague-Dawley rats were used (Holtzman Co., Madison, WI). Starting on day 7 of gestation, dams were fed on a specially prepared diet until the end of gestation. Twenty dams were randomly divided into four groups of five each. Dams of group 1 were fed *ad lib.* a control diet (200 g protein/kg diet). Dams of group 2 were fed on a high-protein diet (400 g protein/kg diet) *ad lib.* Group 3 was pair-fed to group 1 with a 200 g protein/kg diet plus caffeine. Dams of group 4 were pair-fed to group 2 with a 400 g protein/kg diet plus caffeine.

The diets were isoenergetic (Table 1); the increase in dietary protein content was made at the expense of carbohydrate. Caffeine was calculated to provide 20 mg/kg body-weight of dams. To determine the amount of caffeine necessary to provide a dietary content of 20 mg/kg body-weight in groups 3 and 4, average body-weights and daily food intakes of groups 1 and 2 were determined (Yazdani *et al.* 1987). The level of caffeine supplementation to the maternal diet was calculated by taking into account changes in the dams' body-weights and their food intake. Because the dams' body-weights gradually increased during pregnancy, the amount of caffeine in the diets of the caffeine-supplemented groups was adjusted three times over the experimental period to maintain the designated intake. Theoretically the dams received slightly less caffeine toward the end of each given period when the level of caffeine supplementation was recalculated.

Dams were weighed every day, except at weekends. Within 8 h of birth, the pups in each groups were combined and weighed. There is no weight difference attributable to sex at an early age in rats (Nakamoto & Miller, 1977). Three randomly selected pups from each

group were then injected with uniformly labelled [^{14}C]proline (specific activity 273 mCi/mmol; New England Nuclear, Boston, MA) at a dose of 65 μCi/kg. They were then immediately returned on a random basis to a dam within the same group. [^{14}C]proline-injected pups were killed 6 h later, and blood samples were taken in order to determine the precursor pool of [^{14}C]proline. The left and right mandibular bones of each pup were split with a sharp knife, skinned and removed. Tooth germs of molars and incisors and soft tissue were dissected out, together with mandibular nerves from the mandibular bodies, and discarded. The bones were then weighed, and collagen synthesis and total amount of collagen were determined. The procedures were described in detail recently (Nakamoto *et al.* 1987).

Other pups were randomly selected from each group. They were decapitated using scissors and blood was collected and pooled for the determination of plasma caffeine levels. Maternal blood was collected by cardiac puncture into heparinized tubes at the end of the experiment. Bone samples of these pups were removed as described previously. Cleaned mandibles were put in a muffle furnace overnight at 600° and Ca content was measured by atomic absorption spectrophotometry (Nakamoto & Miller, 1977).

Cleaned mandibles, in groups of six, were crushed in a well-chilled porcelain mortar with a porcelain pestle. They were subsequently homogenized in ice-chilled double-distilled water using a glass and Teflon homogenizing apparatus. After homogenization, samples were transferred to test-tubes. After standing for 15 min at 4°, the supernatant fraction was removed and used for protein and enzyme measurements. Total protein in the sample was assayed (Lowry *et al.* 1951). Alkaline (*EC* 3.1.3.1) and acid (*EC* 3.1.3.2) phosphatase activities were measured using a spectrophotometric method (Kuftinec & Miller, 1972).

The caffeine content of plasma samples was measured as follows. Caffeine determinations were performed by HPLC using a Beckman model 344 gradient HPLC system with a 20 μl sample loop and a model no. 427 integrator. The system contained a Supelco C$_{18}$ pre-column and an Altex 46 × 250 mm Ultrasphere ODS reverse-phase C$_{18}$ column. The model 160 absorbance detector was set at a wavelength of 280 nm and run at 0·01 absorbance units full scale.

Samples were eluted over a 13 min time-period with a gradient of 100–400 ml methanol/l water at a flow-rate of 2 ml/min. Three different levels of caffeine and an internal standard were used to calibrate the instrument. The internal standard, β-hydroxy-ethyl theophylline, and the caffeine standard were obtained from Sigma Chemical Co. (St Louis, MO). The methanol and water were HPLC grade.

Extraction was initiated by adding 200 μl of each sample of rat plasma to 1 ml absolute methanol, which precipitated protein. This contained 0·5 μg of the internal standard. The extraction tubes were vortexed for 30 s and centrifuged at 4500 rev./min for 10 min. The supernatant fraction was subsequently transferred to 1·2 × 75 mm tubes, and evaporated under nitrogen in a water-bath at 30°. The residue was prepared for injection by reconstituting in 200 μl methanol (100 ml/l water).

All values were analysed using two-way analysis of variance and multiple comparison (Student–Newman–Keuls) using an Apple II microcomputer (Apple Corp., Cupertino, CA) with $P < 0.05$ considered significant.

RESULTS

Average daily food intakes for the control (group 1) and high-protein (group 2) dietary group were 18 (SE 1) and 20 (SE 1) g respectively. Average food intakes for the caffeine-supplemented control (group 3) and high-protein (group 4) dietary groups were 17 (SE 1) and 20 (SE 1) g respectively. The average number of offspring in each group was as follows:

Table 2. *Caffeine contents ($\mu g/ml$)* in plasma of dams and pups fed on caffeine-supplemented (20 mg/kg body-weight) control (200 g protein/kg) and high-protein (400 g protein/kg) diets†*

(Mean values with their standard errors)

No. of measurements...	Maternal plasma‡ 4	Pups' plasma§ 4
200 g protein/kg diet plus caffeine		
Mean	3·089	2·642
SEM	1·026	1·040
400 g protein/kg diet plus caffeine		
Mean	1·310	1·111
SEM	0·700	0·658

* Pups' plasma for measurement of caffeine content was collected no later than 13 h after birth. Dam's plasma was collected no later than 16 h after delivery.
† For details, see Table 1 and p. 286.
‡ Each dam's plasma was analysed for caffeine content.
§ For each litter plasma from pups that had not been given an injection of [^{14}C]proline was pooled to measure caffeine content.

group 1, 10 (SE 2); group 2, 10 (SE 1); group 3, 8 (SE 1); group 4, 9 (SE 1). There was no statistically significant difference in the number of fetuses among the groups.

The volume of blood plasma collected from one litter of both the caffeine-supplemented control and the high-protein dietary groups was insufficient to measure caffeine, so the caffeine levels of the respective dams were not determined. The mean plasma caffeine concentrations in both dams and pups in the caffeine-supplemented high-protein dietary group were lower than in the caffeine-supplemented control group, but, because of the very large variances, the differences were not statistically significant (Table 2).

There were no statistically significant differences in the birth weights of the groups, although there was a tendency for the high-protein diet groups to have higher body-weights and for caffeine to lower body-weights (Table 3). Mandibular weights, Ca contents and alkaline and acid phosphatase activities of the group fed on a caffeine-supplemented control diet were lower than those of the corresponding unsupplemented group. Ca and protein contents of the group fed on the caffeine-supplemented high-protein diet were lower than those of the corresponding unsupplemented group. On the other hand, alkaline and acid phosphatase activities, collagen synthesis and hydroxyproline contents of the group fed on the caffeine-supplemented high-protein diet were greater than those of the corresponding unsupplemented group (Table 3).

Alkaline and acid phosphatase activities of the group fed on the high-protein diet were lower than those of the control group, whereas the protein levels of the group fed on the high-protein diet were higher than those of the control group. Mandibular weights, alkaline and acid phosphatase activities and collagen synthesis of the group fed on the caffeine-supplemented high-protein diet were higher than those of the group fed on the caffeine-supplemented control diet (Table 3).

DISCUSSION

Assuming the average caffeine content of a cup of coffee to be 100 mg, the present level of caffeine supplementation of the maternal diet is equivalent to a daily intake of ten cups of coffee for a 50 kg female (Gilbert & Pistey, 1973).

Table 3. *Variables measured in the mandibles of newborn pups of dams fed on caffeine-supplemented (20 mg/kg body-weight; C) and unsupplemented (NC) control (200 protein/kg) and high-protein (400 g protein/kg) diets§*

(Mean values with their standard errors)

No. of measurements...	Body-wt (g)‖ 5	Mandibular wt (mg)¶† 6	Calcium (mg) 6	AlP (IU/mg protein) 6	AcP (IU/mg protein) 6	RSA** 5	Hydroxyproline (μmol) 5	Protein†† (mg) 6
200 g protein/kg diet								
NC Mean	7·0	4·85	2·47	0·982	0·058	0·087	0·583	0·21
SEM	0·2	0·03	0·30	0·094	0·007	0·008	0·062	0·02
C Mean	6·4	4·13*	1·38*	0·582*	0·039*	0·091	0·625	0·25
SEM	0·4	0·05	0·21	0·062	0·002	0·006	0·026	0·03
400 g protein/kg diet								
NC Mean	7·4	5·04	2·36	0·307‡	0·030‡	0·077	0·536	0·30‡
SEM	0·2	0·11	0·22	0·022	0·002	0·010	0·055	0·03
C Mean	6·8	4·96†	1·55*	0·872*†	0·059*†	0·153*†	0·742*	0·21*
SEM	0·3	0·11	0·21	0·075	0·006	0·007	0·068	0·02

AlP, alkaline phosphatase (*EC* 3.1.3.1); AcP, acid phosphatase (*EC* 3.1.3.2); RSA, relative specific activity.

* Mean values were significantly different from those for corresponding NC group at each protein level ($P < 0.05$).

† Mean values were significantly different from those for 200 g protein/kg group C ($P < 0.05$).

‡ Mean values were significantly different from those for 200 g protein/kg group NC ($P < 0.05$).

§ For details, see Table 1 and p. 286.

‖ No. of litters averaged.

¶ Bones weighed after drying at 75° for 24 h.

** Calculated to provide an index of the metabolic conversion of proline by the tissue under consideration relative to that in the precursor pool. RSA, which indicates bone hydroxyproline specific activity divided by the blood proline specific activity, provides an index of the fraction of blood proline incorporated into the bone matrix and then converted to hydroxyproline, as an index of collagen synthesis (Nakamoto *et al.* 1987).

†† Primarily represents the non-collagenous protein.

Graham (1978) reported that 74 % of pregnant females consume caffeine, the mean daily intake being slightly more than two cups of coffee, which at the 99th percentile is equivalent to eight cups of coffee. The increased consumption of soft drinks containing caffeine in the past 10 years suggests that the trend for caffeine intake by individuals must be upward, indicating a need to study the effects on the fetus of caffeine intake during pregnancy.

Caffeine and its metabolites pass freely into the embryo and attain concentrations slightly lower than those in the maternal plasma (Scott, 1983). Caffeine may also be poorly metabolized during pregnancy (Kline & Christensen, 1971), causing an accumulation in the fetal tissue (Henderson & Schenker, 1984).

Plasma caffeine levels showed a wide range among the dams as well as among litters, as is shown by the large standard error of means. Although animals were killed at the same time of day, the high variability within dietary groups could partly be due to the food being eaten at different times. It could be argued that dams and pups should be fasted for a specified time period before blood plasma is collected, to obtain less variable caffeine levels. However, with fast-growing pups and lactating dams and the varying eating habits of individual dams, fasting could result in distortion in various measurements conducted on bones.

Because the mean plasma caffeine concentrations in the high-protein dietary group are so far below those of the control group (although not actually significantly different), further studies are warranted to determine the effects of dietary protein levels on metabolic processes controlling plasma caffeine levels.

The different effects observed in caffeine-supplemented and unsupplemented control and high-protein dietary groups for the variables measured suggest that the effects of caffeine may be modified by different nutritional conditions. It is well known that chemicals and drugs can interact with nutrients, including dietary protein levels (Kato *et al.* 1968; Newberne *et al.* 1978).

Alkaline phosphatase activity is reported to have a close functional association with calcification (Salomon, 1974) and bone growth (McWhinnie, 1975), whereas acid phosphatase activity is known to be related to bone resorption (Marks, 1974). We measured alkaline and acid phosphatase activities to determine whether differences in the activities of these enzymes can provide some indication of Ca content.

A decrease in the ratio of alkaline:acid phosphatase activities, for example, indicates a shift of balance towards resorption and thus a lowering of Ca content. Administration of caffeine lowered Ca content. Therefore, in theory, caffeine should lower the ratio of alkaline:acid phosphatase activity in the caffeine-supplemented group relative to the corresponding unsupplemented group. In the control group there was a decrease of 12 %, whereas in the high-protein dietary group the ratio was increased by 44 %.

The activity ratio of alkaline:acid phosphatase in the unsupplemented high-protein dietary group was about 60 % of that of the unsupplemented control group. However, the total amount of calcium was about the same in these groups. Thus, the hypothesis that measurement of the ratio of alkaline:acid phosphatase activities can provide some indication of Ca content is not supported by the evidence in the present study.

The alkaline phosphatase activity of the metaphyses has been shown to be increased with a high-protein diet in young growing rats (Weiss *et al.* 1981). However, this enzyme was decreased with the high-protein diet in the present study. This discrepancy is not surprising due to the different experimental conditions and the different bones investigated in the present study as compared with those in the study of Weiss *et al.* (1981).

cAMP is reported to increase collagen synthesis in fetal calvaria (Phang & Downing, 1973). Caffeine has been known to increase cAMP by inhibiting phosphodiesterase (*EC* 3.1.4.17) (Butcher & Sutherland, 1962). Thus, it is conceivable that collagen synthesis and

total hydroxyproline content, which reflect collagen levels (Bollet, 1970), were increased in the caffeine-supplemented high-protein group compared with the unsupplemented group.

Protein content, measured by an established method (Lowry *et al.* 1951), represents primarily non-collagenous protein. Although small amounts of collagen may also be included, this is negligible. Thus, increased hydroxyproline content and decreased protein content in the caffeine-supplemented high-protein group, compared with the unsupplemented group, indicates that the composition of the collagenous and non-collagenous portions of the organic matrix of the mandible differed between groups.

In a previous study (Nakamoto & Shaye, 1986), we demonstrated that the effect of caffeine on the mandible of the fetus is altered by a maternal dietary protein deficiency. In the present study, it was demonstrated that a maternal dietary protein excess also interacts with caffeine intake and influences fetal mandibular development. While interspecies extrapolation from rat models to the human must be made with caution, pregnant women are nevertheless frequently exposed to caffeine. Thus, some consideration of the interactions between maternal caffeine intake and dietary protein levels during pregnancy could be important for healthy mandibular development in the fetus.

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