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Maternal Low-Carbohydrate High-Protein Diet Affects Mandibular Growth in Diabetic Newborn Rats (42528)

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Abstract. Dams with 7 pups each were randomly assigned to two different diets. Twelve dams were fed a normal (20%) protein diet and were divided into two groups of 4 and 8 animals. Pups from group 1 ($n = 28$) were injected with citrate buffer as a control. Pups from group 2 ($n = 56$) were injected with streptozotocin. Twelve additional dams were fed a 40% protein diet. They were also divided into two groups of 4 and 8 animals. Pups from group 3 ($n = 28$) were injected with citrate buffer as a control. Pups from group 4 ($n = 56$) were injected with streptozotocin. Forty-eight hours later, diabetic status was determined using Dextrostix. On Day 15, pups were injected with [^{14}C]proline to determine collagen synthesis and ^{45}Ca to study mineralization. After the pups were killed, blood glucose levels were determined. Then mandibles were removed. Milk from each dam was also collected after injection of oxytocin. At the time of killing, blood glucose levels in diabetic pups were less than earlier levels, though still higher than those of controls on either diet. The weights of body and mandible, collagen contents, and the total calcium contents in the diabetic group were in general less than those of the nondiabetic group on the 20 and 40% protein diets. ^{45}Ca uptake in the diabetic group was significantly increased compared with those of the nondiabetic rats on both diets. The percentage reduction in the mandibles of diabetic rats from those of nondiabetic rats on the 40% protein diets was consistently less than that of animals on the 20% protein diets. The higher protein contents of the maternal milk in the 40% protein group may partly be responsible for the smaller impairment of mandibular development in the diabetic over nondiabetic animals. It is concluded that maternal low-carbohydrate high-protein diets will play indirectly a beneficial role in the development of the mandibles of diabetic newborns.

Juvenile diabetes exhibits reduced bone formation, which increases the incidence of bone fracture (1). The decrease in calcium deposition and remodeling in diabetic bones has also been well documented (2, 3). In animal studies, the rate of mineralization and bone weight in diabetic rats is less than that in nondiabetic rats (4, 5). Because insulin stimulates intestinal calcium absorption (6) and amino acid uptake, which increases collagen synthesis (7), certain changes result in bone formation and remodeling in the absence of insulin (8–10). In diabetes, overall collagen production is decreased, resulting in a reduction of collagen mass in skin (11). Furthermore, the structure of collagen appears altered (12).

A low-carbohydrate high-protein diet has been reported to be beneficial in cases of mild diabetes in the human (13) and in adult rats (14). On the other hand, it has been shown that a high-protein diet in nondiabetics causes a decrease in osteogenesis (15).

Since newborn rats grow so rapidly in a short period of time, dietary changes during the neonatal period influence the offsprings' growth more than at any other period in their lifetime. Thus, newborn rats have been shown to be an excellent model to study dietary effects on skeletal development (16). Few studies have been done on the streptozotocin-induced diabetic newborn rats, particularly when dietary effects are considered. Our preliminary study indicated that feeding a low-carbohydrate high-protein diet to dams changed milk composition (17). We conduct the present study to determine how this maternal dietary regimen may possibly influence bone development in the diabetic newborns without exogenous insulin administration.

Materials and Methods. A total of 24 timed-pregnant Sprague–Dawley rats (Holtzman Co., Madison, WI) was purchased from a breeder. Upon delivery, all the pups which were born within 8 hr were mixed and desig-

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nated as Day 1. Dams with 7 randomly assigned pups each were divided into four groups: two control groups, each with 4 dams, and two experimental groups, each with 8 dams. Pups from group 1 (n = 28) were injected ip with citrate buffer and their dams were fed a normal (20%) protein diet. Pups from group 2 (n = 56) were injected ip with 10 mg/100 g body wt in which 100 mg of streptozotocin was dissolved in a 1-ml citrate buffer. Their dams were fed a 20% protein diet. Pups from group 3 (n = 28) were injected with citrate buffer. Their dams were fed a 40% protein diet. Pups from group 4 (n = 56) were injected with the streptozotocin in the same manner as group 2, but dams were fed a 40% protein diet. The diets were isoenergetic between 20 and 40% protein (Table I). The increase in dietary protein content was adjusted to the decrease in carbohydrate content. A 20% protein diet is considered normal for rats (18), whereas a 40% protein diet is a low-carbohydrate high-protein diet. Forty-eight hours after streptozotocin injection, pups were tested for the presence of the diabetic state. Blood collected from the tail vein was tested, using Dextrostix (Miles Laboratory Inc., Elkhart, IN).

The weight of the pups and the food intake of their dams were measured every other day until Day 15 postpartum. The last day the pups subsisted solely on the dam's milk was around Day 15 (18). Therefore, experiments were terminated at this time. A small amount of blood was collected from the tail vein and assayed for glucose content, utilizing the glucose oxidase method (19). Pups from the diabetic group (n = 12) and the control group (n = 12) on a 20 and 40% protein diet were injected with uniformly labeled [^{14}C]proline (New England Nuclear, Boston, MA) at a dose of 6.5 μCi/100 g body wt for collagen synthesis study. Six hours later, pups were killed by decapitation and blood was collected to determine the precursor pool of [^{14}C]proline (16). Mandible was selected to study bone growth in the present study. The left and right mandibles of each pup were separated by splitting them in the middle with a sharp knife. The developing molars, incisors, soft tissue, and mandibular nerves from the mandibular body were removed. Cleanliness of the mandibular body was checked under a 10× hand magnifying glass (20).

TABLE I. COMPOSITION OF DIETS (g)

	20% Protein	40% Protein
Casein	200	400
Dextrose	192	122
Sucrose	178	118
Dextrin	192	122
Mazola Corn Oil	150 ml	150 ml
Mineral Mix[a]	40	40
Choline chloride (50%, w/v)	4 ml	4 ml
Cellulose	35	35
Vitamin Mix[b]	10	10

[a] Roger–Harper Mineral Mix (Teklad Test Diets, Madison, WI).

[b] AIN Vitamin Mixture 76 (ICN Pharmaceuticals, Inc., Cleveland, OH).

The specimens were weighed and dried overnight at 110°C. Three randomly selected mandibles were placed in 10-ml ampoules containing 1.5 ml of 6 N HCl, thereby eliminating maternal differences as a source of error and providing a large enough sample to complete the analysis. Each ampoule was flushed with nitrogen for 30 sec, then sealed, and placed in an oven at 125°C for 24 hr. After cooling, the hydrolysates were filtered through a fritted disk funnel. One-milliliter aliquots were taken and diluted with 3 ml of a sodium acetate–citrate buffer at pH 6. Six milliliters of a 5% Na_2CO_2 solution was added to neutralize the acid. Total proline, total hydroxyproline, and the specific activities of [^{14}C]proline and [^{14}C]hydroxyproline were measured as described (16, 21).

Pups in the diabetic groups (n = 10 on 20% protein; n = 12 on 40% protein) and controls (n = 8 on both diets) in 20 and 40% protein diet groups were injected with ^{45}Ca at a dose of 25 μCi/100 g body wt. They were killed 1½ hr later. The mandible was removed as described above. A cleaned bone was placed in a vial containing 0.2 ml of 60% perchloric acid and 0.4 ml of 30% H_2O_2. The vials were then placed in a 75°C oven for 1 hr and oxidized as described (16). A portion of this oxidized sample was subjected to atomic absorption spectrophotometry (Model 280, Fisher Scientific Co., Fair Lawn, NJ) to measure the total calcium content of the bone. The remaining sample was added to 10 ml of scintillation fluid and ^{45}Ca was counted in a liquid scintillation counter (Beckman Model 65-3145 T, Beckman Instruments, Irvine, CA) (16).

After killing all the pups, the dams' milk was collected and assayed for protein, calcium, and phosphorus contents. Each dam was anesthetized with ether and injected ip with 2 IU of oxytocin (22). Milk was collected and frozen until further analysis. When the milk was defrosted at room temperature, a portion of the milk was placed in a porcelain crucible and ashed at 450°C in a furnace for 12 hr (23). The residue was dissolved in concentrated HCl. This solution was then assayed for calcium by atomic absorption spectrophotometry and phosphorus by the method of Fiske and Subbarow (24). Protein was measured by diluting each milk sample with distilled water and assayed by the method of Lowry *et al.* (25). Data were analyzed using analysis of variance and multiple comparison (Student–Newman–Keuls) and Student's *t* test with 5% considered significant. Analyses were performed on an Apple II microcomputer (Apple Corp., Cupertino, CA).

Results. Forty-eight hours after the injection of streptozotocin, the majority of pups showed that blood glucose levels measured by the Dextrostix were more than 250 mg/dl. On the other hand, the nondiabetic group showed glucose limits: 90 mg/dl. However, newborns injected with streptozotocin resulted in approximately 50% mortality compared to only 0.5% in the citrate buffer group. By Day 15, blood glucose levels in both diabetic groups were reduced. However, the 20% + D and 40% + D groups showed significantly higher values than those of the 20% + ND and 40% + ND, respectively ($P < 0.05$) (Table II).

The body growth of the streptozotocin-injected pups was slower than that of the controls in both the 20 and 40% protein diet groups (Fig. 1). By Day 15, the body weights of both diabetic groups were significantly lower than those of the nondiabetic groups on both diets at the end of the experiments ($P < 0.05$) (Table II).

Mandibular weights were significantly lower in both diabetic groups than those of the nondiabetic groups, respectively ($P < 0.05$).

Collagen synthesis in the mandible of the 20% + D group was less than that of the 20% + ND group ($P < 0.05$). Collagen synthesis in the mandible of the 40% + D group was greater than that of the 20% + D group (Table II).

The total hydroxyproline contents of the mandible of the 20% + D group were less than those of the nondiabetic group ($P < 0.05$). Furthermore, the total hydroxyproline contents of mandible in the 40% + D group were greater than those of the 20% + D group ($P < 0.05$). (Table II).

^{45}Ca uptake of mandible of the 20% + D and 40% + D group was greater than that of the nondiabetic groups on the respective diets ($P < 0.05$). ^{45}Ca uptake of mandibles in the 40% + ND group was greater than that in the 20% + ND group ($P < 0.05$) (Table II).

The total calcium contents of the mandible in 20% + D and 40% + D groups were less than those of the nondiabetic groups on respective diets ($P < 0.05$) (Table II).

Calcium and phosphorus contents of milk showed no difference between the 20% protein diet fed dams and the 40% protein diet fed dams ($P > 0.05$). However, the concentration of the protein contents of milk from dams receiving the 40% protein diet was significantly greater than that of the dams receiving the 20% protein diet ($P < 0.05$) (Table III).

Discussion. Prolonged streptozotocin-induced diabetes in the adult rat has been reported to result in a reduced bone turnover (8), but can be corrected by insulin treatment (9). Likewise, decreased bone formation is shown in weanling young male diabetic rats, but can also be corrected by insulin treatment (10). No nutritional study on the bone formation of the diabetic newborn rat is reported. Since the primary purpose of the present study was to determine the nutritional effects, if any, on bone development in diabetic neonates, we did not administer exogenous insulin.

It has been shown that neonates become diabetic after injection of streptozotocin which results in a low amount of pancreatic insulin. Furthermore, high mortality rates (30–50%) in the streptozotocin-injected newborn rats have been reported (26). We have also experienced about 50% mortality in the present study.

Pups in smaller litters are known to grow faster than those in larger litters. Whenever the death of a pup occurred during the experimental period, we replaced it with another which was born the same day and treated in the exact same manner. This procedure was adopted in order to keep litter size constant for each dam. Therefore, as a source of pups we randomly assigned a large number of dams to the streptozotocin-injected groups.

TABLE II. BLOOD GLUCOSE LEVELS, BODY WEIGHT, MANDIBLE WEIGHT, AND VARIOUS PARAMETERS OF BONE METABOLISM STUDIED ON NEWBORN RATS AT DAY 15

	Blood glucose (mg/dl)	Body weight (g)	Weight (mg)	Relative specific activity (RSA)[b]	Hydroxyproline (μmole)	Ca uptake $\left(\frac{\text{dpm} \times 10^3}{\text{Ca(mg)}}\right)$	Calcium (mg)
20% Protein							
Nondiabetic (ND) (group 1)	118.0 ± 1.5 (20)[a]	37.3 ± 0.8 (28)	70.3 ± 3.1 (8)	0.243 ± 0.004 (8)	4.97 ± 0.14 (8)	7.6 ± 0.3 (8)	6.9 ± 0.4 (8)
Diabetic (D) (group 2)	137.2 ± 1.8[d] (19)	29.2 ± 0.9[d] (28)	54.2 ± 2.4[d] (8)	0.211 ± 0.007[d] (8)	4.30 ± 0.13[d] (8)	18.9 ± 0.8[d] (10)	4.9 ± 0.1[d] (10)
40% Protein							
Nondiabetic (ND) (group 3)	121.4 ± 1.9 (19)	36.8 ± 0.5 (28)	68.0 ± 2.2 (8)	0.248 ± 0.008 (8)	5.43 ± 0.23 (8)	11.0 ± 0.3[c] (8)	6.7 ± 0.4 (8)
Diabetic (D) (group 4)	136.9 ± 1.9[d] (20)	29.5 ± 1.2[d] (28)	56.7 ± 1.4[d] (10)	0.240 ± 0.007[c] (8)	5.40 ± 0.22[c] (8)	20.2 ± 0.7[d] (12)	5.3 ± 0.2[d] (12)

Note. Each value represents mean ± SEM.

[a] Values in the parentheses represent the number measured.

[b] Relative specific activity (RSA). RSA was calculated to provide an index of the uptake and/or metabolic conversion of proline by the bone relative to that in the precursor pool as described previously (16). RSA = bone hydroxyproline specific activity divided by the blood proline specific activity. Thus, current RSA provides an index of the fraction of blood proline incorporated into the bone matrix and converted to hydroxyproline, as an index of collagen synthesis. Statistical differences were determined among the groups using analysis of variance (f-test and multiple comparison (Student–Newman–Keul).

[c] Significant difference due to nutritional effect alone ($P < 0.05$) (i.e., group 1 vs group 3; group 2 vs group 4).

[d] Significant difference due to diabetic effect alone ($P < 0.05$) (i.e., group 1 vs group 2; group 3 vs group 4).

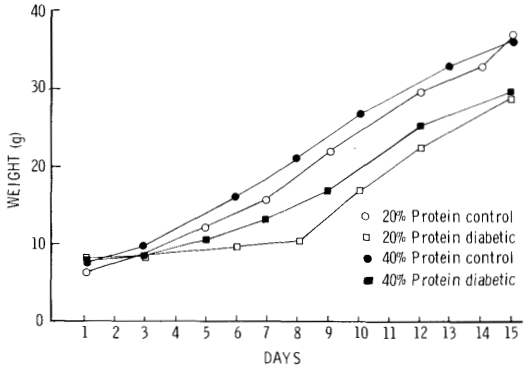

FIG. 1. Represents the changes in body weight of a different group over an experimental period. Each value represents an average of 21 to 28 measurements.

Surviving newborn pups showed in general extremely high levels of blood glucose after 48 hr of streptozotocin injection. However, blood glucose levels were reduced substantially by Day 15. This apparent recovery of blood glucose has been well documented (26, 27). However, these rats remain unable to respond to a glucose challenge (26).

The body weights of the diabetic rats on both diets were about 20% less than those of the respective nondiabetic rats by Day 15, suggesting that the maternal protein levels did not influence the body growth of diabetic newborns.

The weights of the mandibles in the diabetics of the 20% protein group were 23% less than those in the nondiabetic group. On the other hand, mandibular weights in the diabetic rats in the 40% protein group were 17% less than those of the nondiabetic rats. This may suggest that the weight of the mandible in newborns is beneficially affected by a maternal high-protein diet.

Bones consist of two parts (28): an organic part consisting of collagen, ground substance, different types of cells, and enzymes and a mineral part (i.e., hydroxyapatite) in which calcium and phosphate are the main components.

The amount of hydroxyproline, an indicator of collagen content (29), was in general greater in the 40% protein group than in the 20% protein group, whether diabetes was present or not. The hydroxyproline difference between diabetic and nondiabetic pups on the 40% protein diet was negligible. This may also suggest that the organic part of mandible of the newborns may be beneficially affected by the intake of the maternal low-carbohydrate high-protein diet.

Diabetes causes osteoporosis of bones (30). In the animal study, ^{45}Ca uptake in the diabetic young adult rats was less than that of the nondiabetics (31). Our results, however, indicate that ^{45}Ca uptake in the diabetic newborn rats on both 20 and 40% protein diets was consistently increased compared to that of the nondiabetics on both diets. The increase in ^{45}Ca uptake in diabetic pups could partly be due to the partial recovery from severe diabetes. On the other hand, the calcium content of the mandibles in both diabetic groups was less than that of the nondiabetic groups. The decrease in calcium content in the diabetics from nondiabetic animals in the 40% protein group was less than that of the 20% protein group, suggesting that bone calcification of the diabetic pups in the 40% protein group may be less impaired than that of the 20% protein group.

The weights, collagen synthesis, collagen content, and total calcium content of the mandibles in the diabetic vs nondiabetic 40% protein groups were consistently less when compared to the analogous 20% protein group. Thus feeding low-carbohydrate high-protein diets to lactating dams seems to be beneficial to the mandibular development of the diabetic suckling rats. Since the rats' nutrient intake came strictly from maternal milk, the 25% higher protein content of the maternal milk in the 40% protein diet group over that of the 20% protein diet group may be partly responsible for the present observation. It is possible, though, that other unknown factors in milk besides those presently measured may have some influence on the present results.

The present study demonstrates that feeding high protein diets to lactating dams benefi-

TABLE III. COMPOSITION OF MATERNAL MILK AT DAY 15

	Calcium (mg/ml)	Phosphorus (mg/ml)	Protein (mg/ml)
20% Protein	5.03 ± 0.57	2.21 ± 0.17	57.3 ± 0.8
40% Protein	4.14 ± 0.53	2.18 ± 0.20	71.9 ± 0.8[a]

Note. Each value represents mean ± SEM and an average of eight determinations.

[a] Significantly different from 20% protein by Student's t test ($P < 0.05$).

cially affects the growth and development of the mandibles in diabetic newborn rats.

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