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Laboratory Investigations

Intrauterine Exposure to a Maternal Low Protein Diet Reduces Adult Bone Mass and Alters Growth Plate Morphology in Rats

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Abstract. Epidemiological studies suggest that poor growth during fetal life and infancy is associated with decreased bone mass in adulthood. However, these observations have not, to date, been corroborated in animal models. To address this issue we evaluated the influence of maternal protein restriction on bone mass and growth plate morphology among the adult offspring, using a rat model. Maternal protein restriction resulted in a reduction in bone area and BMC, but not BMD, among the offspring in late adulthood. The widened epiphyseal growth plate in the protein-restricted offspring is compatible with the programming of cartilage and bone growth by maternal nutrition in early life.

Epidemiological studies from the United States, Sweden, Australia, and Great Britain have shown that poor growth during fetal life and infancy is associated with decreased bone mass in adulthood [1–7]. Thus, a longitudinal study of 21-year-old women born in Bath demonstrated a positive association between weight at 1 year of age and adult bone mineral content [4]. This relationship was found to persist into late adulthood in a population of men and women aged 60–75 years born in Hertfordshire [5]. One interpretation of these findings is that they represent programming of the skeletal growth trajectory by adverse environmental influences during critical periods of early development [7–10].

Animal studies have provided a reproducible laboratory model with which to investigate the mechanisms of programming, and have replicated many of the epidemiological associations found in humans. The feeding of a low protein diet to pregnant rats has been shown in previous studies to produce offspring that exhibit growth retardation in late pregnancy, and subsequently develop functional changes in adulthood, such as hypertension [11], progressive deterioration of renal function [12], and an impaired immune response [13].

We therefore performed a proof-of-concept study to determine whether skeletal growth is programmed by intrauterine dietary restriction in this rat model. This was achieved in two ways. First, the effect of maternal protein restriction on the bone mass of the adult offspring was assessed by dual-energy X-ray absorptiometry (DXA). Second, we evaluated the effects of this intervention on the morphology and dimensions of the epiphyseal growth plate from long bones of these adult offspring.

Materials and Methods

Animals and Experimental Design

All animal experimentation was performed under license from the Home Office in accordance with the Animals Act (1986). A total of 10 adult female Wistar rats was used to generate the 54 offspring studied in this experiment. All rats were bred within the University of Southampton animal facility and were housed in plastic boxes either singularly or in pairs, in rooms maintained at 22°C, with a 12-hour light cycle. Rats had free access to food and water at all times.

Virgin female rats weighing 220–250 g were mated, and on confirmation of conception through the observation of a semen plug on the floor of the mating cage, were singly housed and fed diets containing 180 g casein/kg (control diet) or 90 g casein/kg (low protein diet), as previously described [14]. The diets were manufactured from purified ingredients within the University of Southampton facility, and were balanced in energy content through the addition of carbohydrate to the low protein diet; the full dietary composition has been published elsewhere [15]. Feeding of the diet continued throughout the 22 days of pregnancy, and during this time food intake and maternal body weight were measured daily. At delivery, the rats were transferred to a nonpurified chow diet (CRME, Special Diet Services, UK). All offspring were weighed and the litters

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were culled to a maximum of 8 pups per litter. One rat fed the low protein diet died during delivery of her litter and data for the food intake and weight gain of this animal were excluded from all analyses.

The offspring of the rats were maintained on CRME diet from weaning at 4 weeks of age until death. Animals were otherwise handled only when cages were cleaned on a weekly basis, and when they were weighed every 4 weeks. They were allowed to die without intervention if there was no evidence of pain, distress, or discomfort. Of the 93 offspring, 23 who were born to dams in the low protein group and 31 born to dams in the control group survived longer than 52 weeks of age, and showed no evidence of rapid weight loss or other distress prior to death. Measurements were made of the weight and nose-to-anal length of the offspring at death. We also evaluated the bone mineral content (BMC) of these animals at death by DXA, and performed histological studies of the proximal tibia and distal femur in a subgroup of 16 animals balanced by age within 2 weeks.

Bone Mineral Measurement

Fifty-four offspring (23 from the maternal low protein group and 31 born to control dams) underwent assessment of bone mineral by DXA (Hologic QDR 2000 bone densitometer; small animal software; Hologic Inc, Waltham, Massachusetts, USA). Whole body BMC, area, and areal BMD (g/cm^2) were evaluated using the ultra-high resolution mode (0.254 mm line spacing and 0.127 mm resolution) and a 0.9 mm collimator used for measuring small laboratory animals. Measurements were also obtained of whole body lean and fat mass during the scan. All measurements were performed after death, and with the animal placed in a standardized prone position. The reproducibility of repeated measurement with repositioning was 2.0%.

Collection and Preparation of Bone Specimens

After death, 16 male animals (9 offspring from protein-restricted dams and 7 control offspring) were selected for histological analyses of the proximal tibial growth plate, having been matched for age within 2 weeks after death. Tibiae were removed at autopsy, and fixed in 4% paraformaldehyde for 48 hours. They were subsequently decalcified in a solution of 5% EDTA in 0.1 M Tris at 4°C, which was replaced on a weekly basis or until decalcification was complete. The tibiae were cut transversely across the center of the shaft, and the distal portion was discarded. The proximal portion was cut coronally through the midline, such that two symmetrical anterior portions remained. These bone samples were dehydrated in graded ethanols, cleared in chloroform, and embedded in paraffin wax. Longitudinal sections of 6–12 μm thickness were mounted on poly-1-lysine-coated slides. Weigerts hematoxylin, Alcian blue, and Sirius red were used to distinguish bone matrix from cartilage matrix. Measurements of the width of the growth plate were made from images at ×98 magnification, at intervals of 15 mm, within a central region of 2800 mm width equidistant from the lateral edges of the bone. Two measurements were obtained at each of these points (Fig. 1). The first (**A**) evaluated the width of the cartilaginous region of the growth plate only; the second (**B**) estimated the width of the entire growth plate, including the metaphyseal band of bone. All measurements were made blind to the maternal diet of each animal. Ten measurements were obtained for each growth plate and utilized in the statistical analyses.

Statistical Analyses

Measurements of bone mineral and tibial growth plate width were normally distributed; measurements were compared between the two groups using a nonpaired Student's *t*-test. As individual measurements were not independent, a multilevel random effects model was constructed to account for the dependence of measurements within rats and within mothers. This model takes account of the associations between offspring born to the same dam, before testing whether maternal nutrition induced significant differences in skeletal measures in the offspring [16]. The effects of maternal nutrition were explored after adjusting for gender, body weight, and these within-rat and within-mother influences.

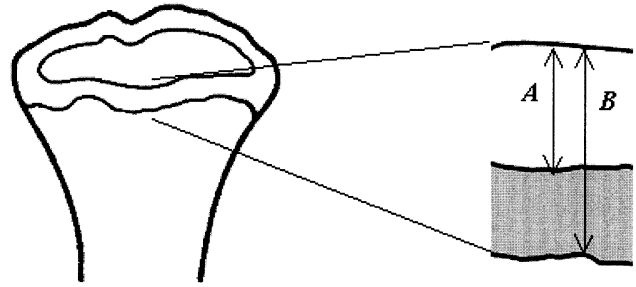

Fig. 1. Measurement criteria for the height of the epiphyseal growth plate. Measurement A was made from the most proximal point of the blue-staining region to the most distal point of this region perpendicular to the axis of the growth plate, thus representing the thickness of the cartilage band. Measurement B was taken to include both the cartilage band and the continuous metaphyseal band of bone. This represented the cumulative thickness of the cartilage band and the metaphyseal band of bone.

Results

Maternal Food Intake, Weight Gain During Pregnancy, and Litter Size

Table 1 shows the maternal food intake and weight gain during pregnancy of the 9 dams studied. Prior to conception, all females were of similar weight (control mean 227, SD 9 g, low protein mean 233, SD 6 g). Pregnancy-associated weight gain was similar in controls and low protein animals over the first 14 days of pregnancy. Between days 15 and 22 of gestation the weight gain of rats fed low protein diets was significantly less than that of the control rats. Food intake tended to be greater in the low protein group throughout pregnancy, but this difference was not statistically significant. Litter size and birth weight did not differ significantly between the two groups [14].

Bone Mineral Measurements

Table 2 and Figure 1 show the differences in whole body bone area, bone mineral content (BMC), and bone mineral density (BMD) between the two groups of animals. There was a statistically significant difference in the mean whole body bone area of the two groups of animals (low protein 53.6 cm^2, control 58.1 cm^2); this difference ($P = 0.01$) in a random effects model allowed for gender, body weight, and animal and mother interrelationships. Thus, the mean bone area of adult offspring born to dams fed low protein diets was around

Table 1. Maternal food intake, weight gain and the outcome of pregnancy

	Maternal diet		
	Control	Low protein	*P*
No. of dams	5	4	–
No. of offspring	31	23	–
Weight gain (g)			
Day 0–7	36 (5)	41 (3)	NS
Day 8–14	44 (3)	37 (4)	NS
Day 15–22	71 (4)	52 (8)	<0.05
Food intake (g/day)			
Day 0–7	27.5 (1.0)	30.0 (1.0)	NS
Day 8–14	28.0 (1.0)	30.0 (1.0)	NS
Day 15–22	27.5 (1.0)	29.0 (3.0)	NS
Litter size (n° pups)	11 (1)	9 (1)	NS
Birth weight (g)	6.01 (0.11)	5.76 (0.12)	NS

All data are shown as mean (SEM). Food intake and weight gain were determined daily throughout pregnancy and are presented as averages for the three separate weeks of gestation. NS = not significant

Table 2. Age, body build, and body composition (using DXA) among adult offspring of dams fed low protein or control diets during gestation

	Maternal diet		*P*-value	
Variable	Control (n = 31)	Low protein (n = 23)	Unadjusted	Adjusted
Age at death (months)	88.1 (4.1)	78.2 (3.0)	0.06	0.06*
Weight at death (g)	405.7 (19.6)	392.5 (18.7)	0.42	0.63*
Length at death (mm)	264.1 (6.3)	256.6 (6.0)	0.41	0.11*
Bone area (cm^2)	58.1 (2.2)	53.6 (2.5)	0.04	0.01†
BMC (g)	9.01 (0.40)	8.29 (0.43)	0.02	0.06†
BMD (g/cm^2)	0.155 (0.003)	0.154 (0.003)	0.98	0.88†
Fat mass (g)	65.1 (8.5)	52.5 (7.4)	0.26	0.22†
Lean mass (g)	285.0 (18.0)	274.5 (16.4)	0.67	0.19†

Figures are mean values with SEM in parentheses
* Difference between groups adjusted for gender only
† Difference between groups adjusted for gender, body weight, animal, and mother inter-relationships, in a random effects model

10% lower than that of offspring born to dams fed a control diet during gestation. A similar magnitude of difference between the two groups was observed for whole body BMC (low protein 8.29 g, control 9.01 g), but the variance in BMC measurements was greater, and the difference between the two groups just failed to attain statistical significance ($P = 0.06$) in the random effects model. Table 2 shows that there was no comparable difference between the groups in whole body BMD ($P = 0.88$). There was a significant positive association ($P < 0.001$) between whole body fat mass and whole body BMC, and when examined in a multivariate model, the adjustment for differences in fat mass were able to eliminate the significant effect of maternal diet on the BMC of the offspring. No such effect was observed for whole body lean mass. The difference between the two groups of offspring in bone area remained after adjusting for both lean and fat mass. Nose-to-anal length, weight, and BMI of the offspring in adulthood did not differ significantly between the two maternal nutrition groups, and the observed differences in BMC remained significant after adjusting for animal length and BMI.

Growth Plate Measurements

The 16 male offspring selected for histological analyses of the proximal tibial growth plate were closely matched for age at death (control group mean age 72.1 weeks, SD 9.4 weeks; low protein group mean age 72.3 weeks, SD 6.7 weeks). Qualitative evaluation of the proximal tibial growth plate preparations from the two groups suggested greater persistence of basal cellular cartilage areas within the growth plates from the low protein group. An example of this change, as well as of difference in growth plate width, is illustrated in Figure 2. Seventy measurements of growth plate width were available from the seven animals in the control group and 90 from the nine

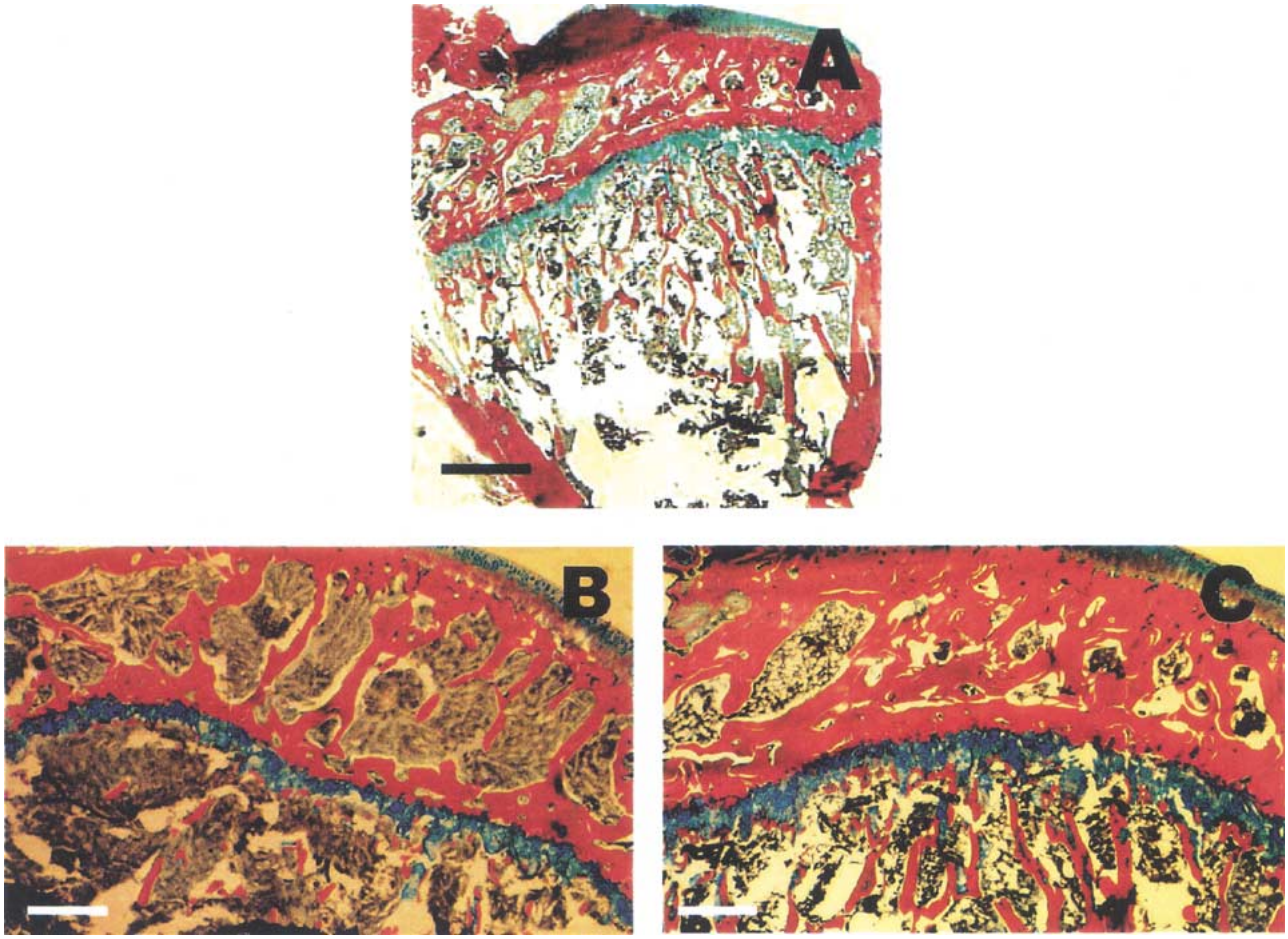

Fig. 2. Histology of rat tibiae. (**A**) Overview of the proximal rat tibia from a 70-week-old male rat, stained with Haematoxylin/Alcian Blue/Sirius Red. The growth plate is seen as a band separating the secondary ossification center from the spongiosa of the diaphysis (original magnification ×35, bar = 1 mm). Comparison of the tibial growth plates from control (**B**) and low protein rats (**C**). In the low protein group, the growth plates were wider, and more spongiosa appeared to be present (original magnification ×35, bar = 0.5 mm).

animals in the low protein group. There were marked differences in the width of the cartilaginous section (measurement A) alone in these two groups of animals (control mean 17 mm, 95% CI 2 mm; low protien mean 22 mm, 95% CI 3 mm). Comparable differences were observed for the width of the entire growth plate, including the adjacent strip of metaphyseal bone (measurement B) (control 25 mm, 95% CI, 2 mm; low protein 36 mm 95% CI 4 mm). These differences were highly statistically significant ($P < 0.001$) when analyzed using a nonpaired *t*-test and remained after Bonferroni correction for multiple testing. Qualitative changes were also observed in the cancellous bone between the growth plate and articular surface (Fig. 2), but these were difficult to quantify. In the random effects model which took account of gender, weight, and effects within and between mothers, the trend towards wider growth plates in the offspring of dams fed a low protein diet persisted, but became non-significant. Detailed measurements of femoral and tibial morphometry were only performed in these 16 animals, and our statistical power to explore differences was correspondingly reduced. There were no significant differences between the two groups regarding tibial or femoral length, width, cortical width, or trabecular bone volume; indeed, the average femoral shaft diameter in the low protein group was slightly greater (mean 210 mm, 95% CI 10 mm) than the diameter in the control group (mean 202 mm, 95% CI 10 mm).

Discussion

The results of this study suggest that maternal protein restriction results in a reduction in bone area and BMC, but not bone mineral density (BMD); among the offspring in late adulthood. These observations in aged rats mirror those made in epidemiological studies among human populations. In addition, we found that maternal protein restriction was associated with changes in the appearance of the epiphyseal growth plate in late adulthood among the offspring. Taken together, these

observations suggest that maternal undernutrition in the rat might program the skeletal growth trajectory by modifying the responsiveness of cells in the growth plate to growth-promoting influences during intrauterine or early postnatal life.

Among the measurements made in the whole group of offspring, the two significant differences observed between the offspring of protein-restricted dams and those of controls lay in bone area and BMC. These suggest that bone size, rather than volumetric bone density, is the principal outcome modified by maternal protein restriction. There were no significant differences between the groups with respect to body weight, nose-to-anal length, or estimated body mass index, suggesting that the dimensions of individual skeletal components (such as femur or tibial length) might be modified by maternal undernutrition. Detailed measurements of individual bones were not performed in this initial proof-of-concept study, but will be included in future investigations. Although highly dependent on lean and fat mass distribution, the differences between the groups in bone area remained after adjusting for these two confounding variables in multivariate models. That for BMC remained after adjusting for lean mass, but was removed by inclusion of fat mass in the model. However, an important weakness of our study lies in the fact that these measurements were performed at natural death, as we were also exploring longevity in the two groups; it is possible that intercurrent illness had selective effects on different body compartments. In future studies we aim to measure bone growth at defined timepoints throughout life. Finally, this study did not include evaluation of a number of potentially programmable systems, such as the kidney or hypothalamic-pituitary axis, which require separate evaluation.

The bone mineral findings support human evidence that the risk of osteoporosis might be modified by environmental influences during early life [7]. Several epidemiological studies have confirmed that infants who are light at birth, and during infancy, have lower adult BMC [1, 2, 4–6]. In addition, a recent Finnish cohort study has demonstrated a direct association between poor childhood growth and later risk of hip fracture [17]. These epidemiological studies are also in accord with follow-up studies of premature infants [18] who appear to have deficits in bone size and mineral content during later childhood, and in whom vitamin D supplementation during infancy has been shown to result in persistent improvements in bone size and mineral density during the pubertal growth spurt [19]. Our data suggest that maternal undernutrition using this rat model provides an experimental system in which the phenomenon of intrauterine programming might be explored further. The diet used in this experiment is not a severe manipulation and could be construed as falling within "normal limits" for the species. Although difficult, extrapolation to the human situation suggests a reduction in maternal protein intake to around 40–50 g daily (well within currently recommended norms) and replacement of this by alternative carbohydrate energy sources.

The histological results of our study suggest that the epiphyseal growth plate might be one site amenable to such programming. The rodent growth plate does not close at skeletal maturity, but during earlier life its thickness is thought to indicate the rate of longitudinal bone growth. Only two previous studies have examined the growth plates from aged rats. Kalu et al. [20] described decreasing thickness and increasing irregularity of the growth cartilage with advancing age in male Fischer rats; Kimmel [21] described ossification within the growth plate and subchondral bony coalescence in aged female Sprague-Dawley rats. In both studies, these changes were small once skeletal maturity had been achieved (estimated at around 16 weeks of age). The influence of maternal undernutrition on the epiphyseal growth plate has not been previously reported.

Our findings of a significantly widened growth plate in the offspring of dams fed a low protein diet during gestation may be interpreted in different ways. First, the observed changes in the width and appearance of the growth cartilage in the low protein group might have been secondary to other metabolic effects of developmental undernutrition, particularly renal disease. Previous studies have reported increased growth plate width, and irregularity at the growth plate-metaphyseal junction, in young rats with experimental renal failure [22]. An alternative interpretation is that they represent direct programming of skeletal growth. This explanation would be consistent with the observations made in human studies, where relationships between weight in infancy and adult bone mass are independent of renal function [5, 6]. This interpretation is also supported by studies of the proliferation of growth plate chondrocytes. During normal aging, the proliferation rate of growth plate chondrocytes diminishes with each successive committed progenitor cell cycle [23]. Glucocorticoid administration to this region supresses proliferation, and the progenitor cells in the affected growth plate of these animals have been shown to undergo fewer cell divisions than those of the control growth plate. Thus, growth plate senescence may be related to the cumulative number of divisions a stem or progenitor cell has undergone. Such a mechanism might permit alteration in growth plate sensitivity to a variety of growth modulating cytokines, for example, 1,25 dihydroxyvitamin D or IGF-1.

Our findings are unlikely to represent an effect of pregnancy protein restriction on maternal milk quality during lactation. The degree of protein restriction utilized in this model is relatively mild, and previous studies have suggested that maternal nutritional status recovers within 24 hours of restoration to a standard

laboratory chow diet. In the present study, animals were not weighed between birth and weaning at 4 weeks, but a previous investigation has demonstrated that the body weights of control and low-protein offspring are similar at 1, 2, 3, and 4 weeks [11].

We conclude that the offspring of rats born to dams fed a low protein diet during pregnancy have reduced bone mineral content, but normal areal bone mineral density. The experimental animals also reveal widened epiphyseal growth plates, an observation compatible with the programming of cartilage and bone growth by maternal undernutrition in early life.

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