The Effect of High Calcium and/or High Protein Diet on Bone Growth in Growing Rats Kept at High Ambient Temperature

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(Received July 6, 1987)

Summary The effects of high calcium and/or high protein diet on bone growth were examined in growing rats kept at high ambient temperature. Thirty-five Wistar strain male rats aged 45 days were divided into 5 groups: room temperature, 24°C and a normal diet (CN), 34°C and a normal diet (HN), 34°C and a high calcium diet (HCa), 34°C and a high protein diet (HPr), and, 34°C and a high calcium-protein diet (HCaPr). The animals were given the same amount of feed. On day 35 of experiment, blood and femurs were collected from all rats. Physical traits, calcium and phosphorus contents of right femur were measured. The number of chondrocyte and the thickness of epiphyseal growth plate were measured in distal epiphysis of left femur. Alkaline phosphatase activities and protein contents were measured in the homogenate of proximal epiphysis and diaphysis of left femur. Ultrafiltrable calcium concentrations and total concentrations of calcium and phosphorus were measured in serum. None of the treatments significantly affected the elongation and densities of calcium and phosphorus in femur. The bone growth in width was reduced at high ambient temperature and high protein diet furthermore reduced the bone growth in width at high ambient temperature. Total calcium and phosphorus concentrations were lower in serum at high ambient temperature. Total calcium concentration in serum was increased by high calcium diet and decreased by high protein diet in hot environment, while ultrafiltrable calcium concentration in serum tended to be higher at high temperature. Since the number of chondrocyte and the thickness of epiphyseal growth plate were greater at high ambient temperature, there was a possibility that the length of femur might become longer at high ambient temperature when the animals were kept at high ambient temperature for a longer period than in the present study.

Key Words bone growth, alkaline phosphatase, epiphyseal growth plate, dietary protein, dietary calcium, high temperature, rat
It was previously reported that the bone growth in width was retarded in rats kept at high ambient temperature though the elongation of bone did not change. And high ambient temperature induced the reduction of calcium content in femur, which was highly associated with the reduction of calcium retention (1). Recently Kume et al. have indicated that calcium requirements increased in cattle kept in hot environment (2).

Holmes (3, 4) reported that the retention of nitrogen was reduced and urinary nitrogen loss was increased in pigs kept at high ambient temperature. Ames and Brink (5) also observed the reduced protein retention in sheep kept at high ambient temperature. Hot environment may increase protein requirements of animals.

In animals raised at high ambient temperature, the retardation of bone growth in width may be caused by the shortage of calcium and/or protein supply. The experiment was conducted to determine the effect of high calcium and/or high protein diet on bone growth in rats reared in hot environment.

MATERIALS AND METHODS

Thirty-five Wistar strain male rats, aged 45 days and averaging 172 g, were divided into 5 groups; room temperature, 24°C and a normal diet (CN), 34°C and a normal diet (HN), 34°C and a high calcium diet (HCa), 34°C and a high protein diet (HPr), 34°C and a high calcium-protein diet (HCaPr), as shown in Table 1. They were fed 11 g of each diet individually and had free access to water, with the condition of 12 h light from 6:00 a.m. The amount of diet was near that of ad libitum feeding at room temperature, 34°C. On day 35 of experiment, blood samples were collected from abdominal aorta of all rats under ether anesthesia during 10:00-12:00. Immediately after slaughter, right and left femurs were collected from all rats. The right femur was boiled in water for 30 min and dried at 110°C for 5 h. The dry weight, volume, length and width of the femur were measured. The right femur was digested with concentrated nitric acid and perchloric acid. Aliquots of the digests were analyzed for calcium and phosphorus contents by an atomic absorption spectrophotometry and the method of Gomori (7), respectively.

In order to observe epiphyseal growth plate, distal epiphysis of left femur was fixed in 10% neutral formalin, demineralized by the method of Plank Rychlo and processed for paraffin-embedded, 5 μm thick sections. The sections were stained with hematoxylin and eosin. Proximal epiphysis and diaphysis of left femur, in which bone marrow was removed, were homogenized in saline with a Biotron (BT20: Biotrona). After centrifugation, alkaline phosphatase activities and protein contents in supernatant were measured by the method of Bessey et al. (8) and Lowry et al. (9), respectively.

Serum calcium and phosphorus concentrations were analyzed by an atomic absorption spectrophotometry and the method of Gomori (7), respectively. After ultrafiltration (CentriFlo CF25: Amicon) with 500 × g, ultrafiltrable calcium concentrations in blood serum were analyzed by an atomic absorption spectro-

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Table 1. Experimental groups and composition of experimental diets.

|                | CN  | HN  | HCa | HPr | HCaPr\(^a\) |
|----------------|-----|-----|-----|-----|-------------|
| No. of animals | 7   | 7   | 7   | 7   | 7           |
| Temperature (°C) | 24  | 34  | 34  | 34  | 34          |
| Protein (%)     | 20  | 20  | 20  | 40  | 40          |
| Ca             | 0.59 | 0.59 | 1.18 | 0.59 | 1.18        |
| P              | 0.56 | 0.56 | 0.85 | 0.60 | 0.85        |
| Cornstarch (g/kg) | 150 | 150 | 150 | 150 | 150         |
| Sucrose        | 485 | 485 | 463 | 287 | 266         |
| Casein         | 200 | 200 | 200 | 400 | 400         |
| Corn oil       | 50  | 50  | 50  | 50  | 50          |
| Cellulose powder | 50  | 50  | 50  | 50  | 50          |
| Mineral mixture\(^b\) | 40  | 40  | 40  | 40  | 40          |
| Vitamin mixture\(^c\) | 20  | 20  | 20  | 20  | 20          |
| CaCO\(_3\)    | 0   | 0   | 5.44 | 3.14 | 10.0       |
| CaHPO\(_4\) \cdot 2H\(_2\)O | 5.43 | 5.43 | 21.83 | 0   | 13.98      |

\(^a\) CN, HN, HCa, HPr, and HCaPr represent control temperature and normal diet, high temperature and normal diet, high temperature and high Ca diet, high temperature and high protein diet, and high temperature and high Ca-protein diet, respectively. \(^b\) Rogers-Harper mineral mixture (6). \(^c\) One gram of vitamin mixture contains 500 IU of vitamin A, 100 IU of vitamin D\(_3\), 5 mg of vitamin E acetate, 5.2 mg of vitamin K, 1.2 mg of vitamin B\(_1\), HCl, 4 mg of vitamin B\(_2\), 0.8 mg of vitamin B\(_6\) HCl, 500 ng of vitamin B\(_12\), 30 mg of vitamin C, 20 μg of d-biotin, 200 μg of folic acid, 5 mg of Ca-pantothenate, 5 mg of p-aminobenzoic acid, 6 mg of nicotinic acid, 6 mg of inositol and 200 mg of choline chloride.

Photometry (10).

Analysis of variance for a 2 × 2 factorial randomized complete block design was performed to test for the effects of high calcium and high protein diets and for the interaction between high calcium and high protein diets at high ambient temperature (34°C). Analysis of variance was performed to test the effects of temperature between CN and HN.

RESULTS

Weight gains of rats during experiment were greater at high ambient temperature than at control temperature, as the same result had been reported previously (7). There was no significant effect of high calcium and/or high protein diet on body weight gains (Table 2).
Table 2. Effects of high ambient temperature, and high Ca and/or high protein diet on weight gains and physical traits of femur.

|                | Weight gain (g/5 weeks) | Dry weight (mg) | Volume (mm³) | Length (mm) | Width (mm) |
|----------------|-------------------------|-----------------|--------------|-------------|------------|
| CN             | 34.1 ± 5.0              | 329 ± 16        | 309 ± 13     | 30.2 ± 0.3  | 3.20 ± 0.16 |
| HN             | 59.9 ± 16.2             | 284 ± 21        | 289 ± 16     | 30.3 ± 0.9  | 3.04 ± 0.08 |
| HCa            | 70.6 ± 9.9              | 294 ± 16        | 283 ± 23     | 30.3 ± 0.6  | 2.99 ± 0.18 |
| HPr            | 66.9 ± 14.1             | 278 ± 26        | 264 ± 19     | 30.4 ± 0.6  | 2.77 ± 0.21 |
| HCaPr          | 60.4 ± 18.5             | 279 ± 7         | 276 ± 15     | 30.3 ± 0.7  | 2.94 ± 0.16 |

Temp. NS
Ca ** NS NS NS NS
Pr NS NS ** NS *
Ca × Pr NS NS NS NS

All values are means ± SE for 7 rats. NS, not significant. *, p<0.05; **, p<0.01.

Table 3. Effects of high ambient temperature, and high Ca and/or high protein diet on calcium and phosphorus contents in femur.

|                | Total amounts | Density |
|----------------|---------------|---------|
|                | Ca (mg)       | P (mg)  |
| CN             | 76.2 ± 5.3    | 34.9 ± 2.6 |
| HN             | 65.9 ± 5.6    | 28.7 ± 2.4 |
| HCa            | 69.4 ± 4.1    | 30.7 ± 3.1 |
| HPr            | 64.6 ± 6.3    | 29.9 ± 1.9 |
| HCaPr          | 65.2 ± 3.0    | 28.7 ± 3.1 |

Temp. ** NS
Ca NS NS NS NS
Pr NS NS NS NS
Ca × Pr NS NS NS NS

All values are means ± SE for 7 rats. NS, not significant. **, p<0.01.

Physical traits of femur are shown in Table 2. Bone growth in width substantially reduced at high ambient temperature though the elongation did not significantly alter. The result coincided with that observed in a previous experiment (1). Although a significant effect of high calcium diet was not observed, the volume and the width of femur were significantly reduced by high protein diet at high ambient temperature.
Table 4. Effects of high ambient temperature, and high Ca and/or high protein diet on enzyme activities in femur, the thickness of epiphyseal growth plate and the number of chondrocyte in epiphyseal growth plate.

|          | AlPase activity | Thickness of growth plate | No. of chondrocyte |
|----------|-----------------|---------------------------|--------------------|
|          | Epiphysis       | Diaphysis                 | (μm)               |                    |
| CN       | 397 ± 56        | 175 ± 24                  | 195 ± 16           | 100 ± 12          |
| HN       | 337 ± 63        | 140 ± 28                  | 243 ± 24           | 138 ± 16          |
| HCa      | 291 ± 96        | 138 ± 45                  | 256 ± 19           | 140 ± 22          |
| HPr      | 341 ± 68        | 152 ± 40                  | 248 ± 16           | 134 ± 19          |
| HCaPr    | 250 ± 35        | 109 ± 22                  | 230 ± 7            | 141 ± 3           |
| Temp.    | NS              | *                         | *                  | **                |
| Ca       | *               | NS                        | NS                 | NS                |
| Pr       | NS              | NS                        | NS                 | NS                |
| Ca × Pr  | NS              | NS                        | NS                 | NS                |

All values are means ± SE for 7a and 5L animals. NS, not significant. *, p < 0.05; **, p < 0.01. Enzyme activities are expressed in mM substrate utilized per mg soluble protein per hour at 37°C. The numbers of chondrocyte are expressed as % to those of CN group.

Calcium and phosphorus contents in a whole bone and on the basis of bone dry weight are presented in Table 3. The contents of calcium and phosphorus reduced at high ambient temperature, which appeared to be associated with the decrease of bone growth in width. The high calcium and/or high protein diet had no significant effect on calcium and phosphorus contents in a whole bone. Any treatments in this study did not significantly affect calcium and phosphorus contents of bone on the basis of bone dry weight.

Alkaline phosphatase activities in soluble fraction of bone homogenate are expressed in mM substrate utilized per mg soluble protein per hour at 37°C (Table 4). Alkaline phosphatase activities in epiphysis were significantly reduced by high calcium diet but were scarcely affected by temperature and by high protein diet. Alkaline phosphatase activities in diaphysis significantly decreased at high ambient temperature but were not influenced by high calcium and/or high protein diet.

The thickness of epiphyseal growth plate and the number of chondrocytes in epiphyseal growth plate were significantly greater at high ambient temperature than at control temperature, but were little affected by high calcium and/or high protein diet (Table 4).

Total calcium concentrations in serum were lower at high ambient temperature than at control temperature. At high ambient temperature, total calcium concentrations were decreased by high protein diet and increased by high calcium diet, while the level at high temperature did not reach the level at control temperature.
Table 5. Effects of high ambient temperature, and high calcium and/or high protein diet on total and ultrafiltrable calcium concentrations and total phosphorus concentration in blood serum.

|                | Total calcium (a) (mg/100 ml) | Ultrafiltrable calcium (b) (mg/100 ml) | (b)/(a) ratio (%) | Total phosphorus (mg/100 ml) |
|----------------|-------------------------------|---------------------------------------|-------------------|-----------------------------|
| CN             | 11.10 ± 0.68                  | 4.21 ± 0.62                           | 38.5 ± 7.5        | 9.12 ± 1.31                 |
| HN             | 10.24 ± 0.39                  | 4.62 ± 0.87                           | 45.3 ± 9.4        | 6.90 ± 0.82                 |
| HCa            | 10.44 ± 0.28                  | 5.13 ± 0.30                           | 49.2 ± 2.7        | 7.15 ± 0.46                 |
| HPr            | 9.82 ± 0.15                   | 4.80 ± 0.70                           | 48.8 ± 6.9        | 7.19 ± 0.57                 |
| HCaPr          | 10.24 ± 0.47                  | 4.77 ± 0.75                           | 46.7 ± 8.4        | 7.73 ± 0.78                 |
| Temp.          | *                             | NS                                    | NS                | **                          |
| Ca             | *                             | NS                                    | NS                | NS                          |
| Pr             | *                             | NS                                    | NS                | NS                          |
| Ca × Pr        | NS                            | NS                                    | NS                | NS                          |

All values are means ± SE for 7 rats. NS, not significant. *, p<0.05; **, p<0.01.

Ultrafiltrable calcium concentrations in serum tended to be higher at high ambient temperature in spite of low total concentrations in serum. The ratio of ultrafiltrable to total calcium concentrations also had a higher tendency at high ambient temperature in comparison with that at control temperature. High calcium and/or high protein diet had no significant effect on ultrafiltrable concentration of calcium and the ratio of ultrafiltrable to total calcium concentration in serum. Phosphorus concentrations in serum were significantly lower at high ambient temperature than at control temperature. The serum phosphorus, however, was little influenced by high calcium and/or high protein diet (Table 5).

**DISCUSSION**

It is generally recognized that thyroid function is reduced in hot environment. The suppressed basal metabolism through the reduction of thyroid function might contribute to the increase in weight gain at high ambient temperature since animals of each group were fed the same amount of diet. Although the animals gained more at high ambient temperature, bone growth in width and bone contents of calcium and phosphorus were suppressed at high ambient temperature.

It was reported that bone alkaline phosphatase activities were closely related to calcification and formation of bone (11–13). Suppressed activities of alkaline phosphatase in diaphysis at high temperature could explain the retardation of bone growth in width of rats at high temperature. But the reason why alkaline phosphatase activity was reduced by high calcium diet at high ambient temperature is not clear.

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Although the length of femur differed little among treatment groups in this experiment, the epiphyseal growth plate was thicker and the number of chondrocyte increased at high ambient temperature when compared with those at control temperature. Therefore, there is a possibility that the elongation of femur may become greater at high ambient temperature than at control temperature when the animals are kept for a longer period than in the present study.

There seemed to be some causes which induced the enlargement of growth plate thickness in hot environment, although the details were not clear. Considering the greater weight gains of animals at high ambient temperature, surplus nutrients might promote the proliferation of chondrocyte in epiphyseal growth plate. On the other hand, Al-Hilli and Wright (14) reported that the tail bones of the mice kept at high ambient temperature grew longer and faster than those in control, suggesting that local factors induced by heat exposure influenced the closure of epiphyseal growth plate in caudal vertebrae.

Some workers indicated that high protein diets resulted in negative calcium balance through the increase of urine calcium excretion in adult rats (15–17), while this did not influence bone constituents (16). In the present study, however, the bone growth in width was retarded by high protein diet although the treatment did not affect the calcium and phosphorus densities based on bone weight in rats reared in hot environment. Therefore, it was suggested that a high protein diet might reduce the growth in width in hot environment. The result in this experiment that serum calcium concentrations significantly decreased in rats fed high protein diets might be, partly at least, caused by the increase of urinary calcium loss.

High calcium diet scarcely influenced bone morphology, although the treatment improved calcium concentrations in serum. Calcium concentrations in serum decreased in hot environment; nevertheless, ultrafiltrable calcium concentrations in serum tended to be higher at high ambient temperature than at control temperature. The reason why the opposite change of serum ultrafiltrable calcium against serum total calcium occurred is not clear. A further study will be necessary to clarify the relationship between serum calcium concentrations and serum ultrafiltrable calcium concentrations in animals kept at high temperature.

The authors are grateful to Prof. Yukio Yamada and Prof. Ryoji Kawashima for their helpful suggestions and encouragements throughout this study.

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