Amino acid supplementation alters bone metabolism during simulated weightlessness

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1Human Adaptation and Countermeasures Office, National Aeronautics and Space Administration Lyndon B. Johnson Space Center, and 2Enterprise Advisory Services, Inc., Houston, Texas; and 3Departments of Surgery

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Submitted 22 December 2004; accepted in final form 28 January 2005

Zwart, S. R., J. E. Davis-Street, D. Paddon-Jones, A. A. Ferrando, R. R. Wolfe, and S. M. Smith. Amino acid supplementation alters bone metabolism during simulated weightlessness. J Appl Physiol 99: 134–140, 2005. First published February 3, 2005; doi:10.1152/japplphysiol.01406.2004.—High-protein and acidogenic diets induce hypercalciuria. Foods or supplements with excess sulfur-containing amino acids increase endogenous sulfuric acid production and therefore have the potential to increase calcium excretion and alter bone metabolism. In this study, effects of an amino acid/carbohydrate supplement on bone resorption were examined during bed rest. Thirteen subjects were divided at random into two groups: a control group (Con, n = 6) and an an amino acid-supplemented group (AA, n = 7) who consumed an extra 49.5 g essential amino acids and 90 g carbohydrate per day for 28 days. Urine was collected for n-telopeptide (NTX), deoxypyridinoline (DPD), calcium, and pH determinations. Bone mineral content was determined and potential renal acid load was calculated. Bone-specific alkaline phosphatase was measured in serum samples collected on day 1 (immediately before bed rest) and on day 28. Potential renal acid load was higher in the AA group than in the Con group during bed rest (P < 0.05). For all subjects, during bed rest urinary NTX and DPD concentrations were greater than pre-bed rest levels (P < 0.05). Urinary NTX and DPD tended to be higher in the AA group (P = 0.073 and P = 0.056, respectively). During bed rest, urinary calcium was greater than baseline levels (P < 0.05) in the AA group but not the Con group. Total bone mineral content was lower after bed rest than before bed rest in the AA group but not the Con group (P < 0.05). During bed rest, urinary pH decreased (P < 0.05), and it was lower in the AA group than the Con group. These data suggest that bone resorption increased, without changes in bone formation, in the AA group.

Bone resorption; methionine; n-telopeptide; acidosis; bed rest

Bone loss and fracture risk are significant concerns for astronauts and the general public. Diet can have a profound effect on acid-base balance of the body, which in turn could affect bone metabolism. Acid-base balance is determined mainly by the partial pressure of carbon dioxide and endogenous acid production from acid components in the diet (7, 21). Net endogenous acid production results from incomplete oxidation of organic acids and production of sulfuric acid by oxidation of sulfur-containing amino acids. In contrast, acid production can be neutralized by sources of bicarbonate in the diet or endogenous sources (7). Vegetables mainly provide base rather than acid precursors; they contain high concentrations of bicarbonate and other organic anions that can be converted to bicarbonate. Bicarbonate is one of the body’s main buffering systems, and bone serves as a major body reservoir for bicarbonate (33).

In vitro studies show that low pH induces bone resorption through a direct physiochemical effect as well as a cellular effect on bone (1). It is currently debated whether excess acid precursors in the diet result in increased bone resorption. Many studies show positive associations of high protein intake with bone turnover (3, 9, 14, 23, 30, 41, 49), whereas others show negative associations (5, 10, 11, 28, 34, 35). These disparate results could be related to differences in the levels of base precursors in the diet, and they are likely also related to calcium intake or status. Some studies show that dietary protein increases calcium absorption, and that this may be the reason why high amounts of dietary protein induce hypercalciuria (16, 17). If protein intake is high and yields increased urinary calcium excretion, then a high-protein diet accompanied by a low calcium intake can result in a negative calcium balance (4, 5). Bed rest is an analog of spaceflight that results in bone resorption and increased urinary calcium excretion from disuse; therefore, ingestion of excess protein or sulfur-containing amino acids during bed rest could exacerbate a negative calcium balance and increase bone resorption. We have previously shown that a higher ratio of animal protein to potassium intake yields increased urinary excretion of calcium and markers of bone resorption during a 30-day bed rest study (54).

Bone loss that is accompanied by decreased calcium absorption and increased calcium excretion is a common problem among astronauts during spaceflight and is a source of increasing concern for long-term missions in the future. Bone loss associated with spaceflight could be exacerbated by a diet that is not balanced between acid and base precursors. This study was primarily designed to assess the ability of an essential amino acid supplement to mitigate loss of muscle mass and strength during bed rest. The primary results have been published (32). In this study, we report the effects of this potential muscle loss countermeasure on bone metabolism and calcium excretion.

Materials and Methods

Subjects. Thirteen male subjects were randomly assigned to an amino acid-supplemented (AA, n = 7) group (36 ± 10 yr, 87 ± 12 kg, 180 ± 3 cm) or control (Con, n = 6) group (38 ± 8 yr, 86 ± 10 kg, 179 ± 3 cm) (values are means ± SD).

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All subjects gave informed, written consent according to the guidelines established by the Institutional Review Boards at the University of Texas Medical Branch and the Johnson Space Center. The study was approved by both of these Boards. Subject eligibility was assessed by a battery of medical screening tests including medical history, physical examination, electrocardiogram, blood count, plasma electrolytes, blood glucose concentration, and liver and renal function tests. Exclusion criteria included recent injury, the presence of a metabolically unstable medical condition, low hematocrit or hemoglobin, vascular disease, hypertension, and cardiac abnormality.

**Bed rest protocol.** Subjects were admitted to the General Clinical Research Center (GCRC) at the University of Texas Medical Branch at Galveston for 5 days of dietary stabilization and pretesting before the start of bed rest. During this period, subjects were sedentary but remained ambulatory. On days 1 and 28 of the bed rest study, a tracer kinetics study was conducted, and muscle biopsies and blood samples were collected as described in detail elsewhere (32).

Beginning on day 1 of bed rest, the AA group consumed supplements containing 16.5 g of essential amino acids three times per day at 1100, 1600, and 2100 (Table 1). Sucrose was included to improve palatability. The supplement was dissolved in a noncaloric soft drink, and the Con group received only the noncaloric soft drink. Subjects remained in strict bed rest from days 1 to 28, and they were continually monitored by GCRC nursing staff.

During daily activities (reading, computer use, television viewing), subjects were allowed to raise their shoulders with two pillows, and a slight bed-back elevation was permitted. Subjects were allowed to change position periodically to alleviate positional discomfort and to eat. Bathing, hygiene activities, and urine collection were performed during bed rest. Subjects were permitted to use a bedside commode for bowel movements, but the time out of bed was limited to ~5 min. After completing poststudy testing (day 29), subjects slowly resumed weight-bearing activities and were discharged after medical evaluation 72 h later.

Blood was collected, by standard phlebotomy techniques, into evacuated blood collection tubes and processed for serum collection. Fasting (8 h) blood samples were collected at 0600 immediately before bed rest on day 1 and on day 28 during bed rest. Urine (24 h) was collected for 2 days before bed rest, daily during bed rest, and for 2 days after bed rest (days 29 and 30). Statistical analyses of these samples were performed on the average of the pre-bed rest data and on weekly averages during bed rest. Incomplete 24-h pools were collected on days 26 and 27 for some of the subjects, and these samples were not used in the analysis. One subject had only one urine sample on bed rest day 22; thus no week 4 mean was calculated for that subject. Baseline urine samples were missing from one subject in the Con group, and this subject was removed from these analyses.

**Dietary intake.** During the diet stabilization period and for the duration of the study, subjects were placed on a diet with a 3-day menu cycle. The daily energy requirement for each individual was determined by using the Harris-Benedict equation with an activity factor of 1.6 (pre-bed rest diet stabilization period) or 1.3 (bed rest). Daily nutrient intake was evenly distributed among three meals (0830, 1300, 1830), with carbohydrate, fat, and protein representing 59, 27, and 14%, respectively, of daily intake. Water was provided ad libitum, and none of the subjects consumed caffeine during the study. Nutrient calculations were performed for one representative 3-day menu cycle using the Nutrition System Database for Research version 4.06.34, developed by the Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN, Food and Nutrient Database 34 released May 2003 (40). Net acid excretion (NAE) was determined indirectly from the difference between the sum of the major urinary nonbicarbonate anions (chloride, phosphate, sulfate, and organic acids) and the sum of the urinary nontitratable acid and non-NH₃ cations (sodium, potassium, magnesium, and calcium). The potential renal acid load (PRAL) comprises the dietary component of the NAE estimation, and corrects for intestinal absorption of minerals, methionine, and cysteine. Organic acid (OA) excretion is largely determined by body surface area (BSA) (22, 36, 37), which was determined in this study using the Mosteller formula (27).

The calculation for estimating NAE (meq/day) was adapted from Remer and colleagues:

$$\text{NAE} = \text{PRAL} + \text{OA} \quad (36, 38)$$

where

$$\text{PRAL} = [2 \times (0.00503 \times \text{mg methionine/day}) + (0.0062 \times \text{mg cysteine/day}) + (0.037 \times \text{mg phosphorus/day}) - (0.021 \times \text{mg potassium/day}) - (0.026 \times \text{mg magnesium/day}) - (0.013 \times \text{mg calcium/day})] \quad (8, 36, 38)$$

$$\text{OA} = \text{BSA} \times 1.73 \quad (22, 36, 37)$$

$$\text{BSA} = [(\text{cm height} \times \text{kg weight})/3,600]^{0.42} \quad (27)$$

The conversion factors for PRAL are based on the percent absorption (reviewed in Ref. 38) divided by the respective atomic weight. To estimate meq SO₄ (= mmol SO₄ × 2) (8), we assumed the absorption of methionine and cysteine from protein and the supplement to be 75% for both, because we did not expect the absorption of protein-bound methionine and free methionine (26, 42) to be different.

**Biochemical analyses.** The collagen cross-links n-telopeptide (NTX) and deoxyypyridinoline (DPD) in urine were measured with commercially available ELISA kits (Quidel, Santa Clara, CA, and Ostex International, Seattle, WA, respectively), as described previously (43). Urinary calcium was measured by inductively coupled plasma emission mass spectrophotometry techniques, as described previously (43). Urine pH was also determined (Thermo Orion pH meter, Beverly, MA). Serum bone-specific alkaline phosphatase (BSAP) was measured by a commercially available ELISA (Quidel). Urinary creatinine was measured as previously described (44).

Bone mineral content (BMC) was determined on days 2 or 3 of diet stabilization before bed rest, during bed rest (day 14), and after bed rest.
The biochemical analyses, weekly averages were determined for each supplemented (AA, Con) groups before (Pre) and during 4 wk of bed rest. A Day; wk, week. *Main effect of time: significantly different from baseline values, $P < 0.05$ (no significant difference between groups).

**RESULTS**

Energy intake was 3,040 $\pm$ 228 and 2,420 $\pm$ 193 kcal per day for the AA and Con groups, respectively. Total protein intake was 135 $\pm$ 7 g for the AA group and 87 $\pm$ 7 g for the Con group. As expected, energy and total protein intake for the AA subjects were significantly greater than those of Con subjects ($P < 0.05$). Calcium intake was 720 $\pm$ 139 and 719 $\pm$ 157 mg for the AA and Con groups, respectively. Sodium intake was 3,635 $\pm$ 618 and 3,675 $\pm$ 536 mg/day for the AA and Con groups, respectively. Phosphorus intake was 1,363 $\pm$ 232 and 1,356 $\pm$ 254 mg/day for the AA and Con groups, respectively. Intakes of calcium, sodium, and phosphorus were not significantly different between groups.

Urinary NTX (Fig. 1) and DPD (Fig. 2) excretion were significantly elevated from baseline during all weeks of bed rest for all subjects ($P < 0.05$, main effect). Although urinary NTX was generally higher in the AA group than in the Con group (Fig. 1), the difference was not statistically significant ($P = 0.073$). DPD also tended to be higher in the AA group compared with the Con group during bed rest ($P = 0.056$).

Dietary sulfur was computed from methionine and cysteine content in the diet for all subjects (8) and was positively correlated ($r = 0.62, P < 0.05$) with urinary NTX excretion during the last week of bed rest (Fig. 3). Similar results were also found for the third week of bed rest ($r = 0.63, P < 0.05$). There was no significant relationship between dietary sulfur and urinary calcium or pH.

Net acid excretion estimated from organic acid excretion and dietary sulfur was higher in the AA group than in the Con group (57.4 $\pm$ 13.3 and 43.0 $\pm$ 7.2 meq/day for the AA and Con groups, respectively, $P = 0.055$). Potential renal acid load was significantly higher in the AA group compared with the Con group (8.1 $\pm$ 10.0 and $-5.1 \pm 5.1$ meq/day, respectively, $P < 0.05$). Net acid excretion was also estimated by using calculations developed by Remer and colleagues (38), in which sulfate excretion is estimated from total protein intake. In the calculations developed by Remer and colleagues, an average content of 2.4% methionine and 2.0% cysteine is assumed, and...
In the AA group, total BMC was significantly lower after bed rest than before bed rest (2,493 ± 404 and 2,524 ± 387 g/cm² respectively, P < 0.05), but in the Con group, BMCs before and after bed rest (2,267 ± 330 and 2,257 ± 321 g/cm², respectively) were not significantly different from each other. Body weight for the AA group was not different after bed rest compared with before (86.8 ± 4.7 and 86.9 ± 4.7 kg, respectively), but body weight was less after bed rest for the Con group (86.1 ± 4.3 kg before bed rest and 83.7 ± 3.9 kg after bed rest, P < 0.01). Detailed findings regarding body composition and muscle strength changes from this study have been described elsewhere (32).

**DISCUSSION**

This experiment was originally designed to test the ability of an amino acid supplement to minimize muscle loss during bed rest. A secondary hypothesis was that maintaining muscle mass would help to mitigate the bone and calcium loss normally observed in bed rest. We found that although taking the supplement maintained muscle mass (32), urinary excretion of calcium, NTX, and DPD were greater in the AA group than in the controls, and urinary pH and BMC were lower in the AA group than in the controls. Because the only difference between the two groups was the dietary supplement, these findings suggest that some aspect of the proposed countermeasure contributed to increased bone resorption and calcium loss. Differences in urine volume and urinary creatinine excretion between the two groups were not significant, so it is not likely that a change in clearance could explain the increase in markers of bone resorption and change in urine pH. The fact that the difference in serum BSAP between the groups was not significant suggests that the supplement increased resorption without changing bone formation.

It is possible that the supplement induced chronic mild metabolic acidosis, which contributed to increased bone resorption. If the kidney is functioning properly and excess acid is excreted in the urine, then the diet has the potential to decrease urine pH if more acid than base precursors are ingested (3, 6, 24, 37). Sulfur is predominantly responsible for determining the net endogenous acid production from protein because it is the acid precursor that is oxidized to sulfuric acid (8). It would therefore make sense that a dietary supplement with excess sulfur-containing amino acids could yield in-

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**Table 2. Urinary excretion of collagen cross-links before and during bed rest**

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
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</thead>
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<tr>
<td><strong>Urine volume, ml/day</strong></td>
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<tr>
<td>AA</td>
<td>3,335 ± 738</td>
<td>3,147 ± 445</td>
<td>2,889 ± 839</td>
<td>2,992 ± 841</td>
<td>3,021 ± 1,107</td>
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<tr>
<td>Con</td>
<td>3,040 ± 1,045</td>
<td>3,434 ± 794</td>
<td>3,269 ± 1,176</td>
<td>3,274 ± 1,203</td>
<td>3,215 ± 1,224</td>
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<td><strong>Urinary creatinine, mmol/day</strong></td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>AA</td>
<td>20 ± 4</td>
<td>19 ± 3</td>
<td>20 ± 3</td>
<td>19 ± 3</td>
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<tr>
<td>Con</td>
<td>18 ± 5</td>
<td>18 ± 6</td>
<td>18 ± 4</td>
<td>17 ± 4</td>
<td>17 ± 4</td>
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<tr>
<td><strong>NTX, nmol/mmol creatinine</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>AA</td>
<td>28.5 ± 7.4</td>
<td>39.0 ± 14.3*</td>
<td>43.1 ± 17.5*</td>
<td>46.2 ± 14.1*</td>
<td>46.2 ± 15.2*</td>
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<tr>
<td>Con</td>
<td>27.2 ± 6.6</td>
<td>31.6 ± 4.6*</td>
<td>33.0 ± 5.7*</td>
<td>36.3 ± 6.0*</td>
<td>37.8 ± 7.3*</td>
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<td><strong>DPD, nmol/mmol creatinine</strong></td>
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<td></td>
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<td>AA</td>
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<td>4.3 ± 1.2*</td>
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<td>5.3 ± 1.5*</td>
<td>5.4 ± 1.8*</td>
</tr>
<tr>
<td>Con</td>
<td>3.4 ± 0.7</td>
<td>4.3 ± 1.1*</td>
<td>4.3 ± 1.1*</td>
<td>4.7 ± 1.2*</td>
<td>4.9 ± 1.7*</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 6 (Con) or 7 (AA). AA, amino acid-supplemented group; Con, placebo group; NTX, n-telopeptide; DPD, deoxypyridinoline. *Main effect of time: significantly different from baseline, P < 0.05 (no significant difference between groups).
creased sulfuric acid production in the body. The amino acid supplemented group received a total of 3.5 ± 0.2 g methionine per day, equivalent to ~250% of the recommended daily intake (29). Because the AA and Con groups consumed identical diets each day (besides the dietary supplement), we must assume that endogenous acid production resulting from diet alone was also similar for the two groups. Several other human and animal studies have shown that methionine supplementation alone elicits similar responses: decreased bone density (51), increased urine calcium excretion (19, 48, 50), and acidified urine (13, 25). In this study, dietary sulfur (calculated from the sulfur content of amino acids in the diet) was correlated with urinary NTX excretion. Specifically, the more sulfur the diet contained, the more NTX was excreted, which supports a negative effect of excess dietary sulfur on bone.

The effect of excess protein intake on bone metabolism is not well understood, as shown by the many studies with conflicting results. Several recent studies show that high protein intake has a beneficial effect on bone (15, 39), but these studies also have factors that potentially confound the results. For instance, the groups receiving higher protein diets generally also consume higher amounts of dietary phosphorus. Dietary phosphorus has been found to mitigate protein-induced hypercalciuria (46, 47, 52, 53). It is suggested that the high phosphorus content of most foodstuffs containing dietary protein may inhibit protein-induced hypercalciuria and bone loss (12, 52). Another recent study comparing dietary protein levels and bone metabolism maintained constant dietary phosphorus contents among groups, and they found that higher dietary protein intake was associated with decreased net renal acid excretion, decreased calciuria, and decreased markers of bone resorption (12). The present study describes the effect of a dietary amino acid and carbohydrate supplement, whereas all other dietary parameters, including phosphorus, were kept constant between treatment groups. The results of the present study support the hypothesis that excess protein (or sulfur-containing amino acids) in the presence of inactivity (30 days of bed rest) yields an overall negative calcium balance and increased bone resorption. We do not know whether intestinal calcium absorption was increased by the supplement, but the increased excretion of calcium by the AA group, along with increased excretion of bone resorption markers and decreased BMC without an increase in bone formation, support a negative calcium balance. Bed rest provides a unique model to examine the effects of excess dietary protein on bone metabolism because calcium balance is typically lower as a result of increased excretion and decreased absorption (18). During spaceflight, calcium balance is also low as a result of decreased calcium absorption and increased excretion. The results from the present bed rest study suggest that excess dietary protein without sufficient base precursor intake during spaceflight may exacerbate the typical increases in bone resorption and calcium excretion.

Although this study has some limitations that constrain the conclusions that can be drawn, these data do support the thesis that acidosis and bone resorption can result from excess protein intake, given in the form of an essential amino acid mixture containing methionine. If methionine alone had been used as an amino acid supplement instead of a mixture, a definitive role for the sulfur-containing amino acids could be identified. Although it would have been ideal to have energy intake and body weight the same in both groups, we would not expect this to increase bone resorption without changing bone formation. The small decrease in body weight in the Con group was not accompanied by a change in BMC or a change in bone formation markers. It is also not clear why no change in urinary calcium occurred in the control subjects during bed rest. Although we cannot rule out the possibility that the carbohydrate in the supplement contributed to the hypercalciuria in the AA group (31), we would not expect the carbohydrate to affect urinary pH or total body BMC. Furthermore, even though one study shows that aspartame can induce calciuria (31), that was not the case in this study because both groups received the same amount of aspartame, and urine calcium was increased only in the AA group. It is noteworthy that calcium intake during bed rest was consistently lower than the recommended dietary intake of calcium, which could contribute to the lack of change in urinary calcium in the Con group. Although the calcium excretion in the Con group did not increase as expected, it is notable that the change in cross-link excretion in the Con bed rest group of this study was of a magnitude similar to that observed in previous bed rest studies of similar duration (2, 20, 43, 45). Furthermore, the difference in urinary calcium excretion between the Con and AA groups before bed rest was not significant.

Despite the limitations of the study, these data highlight the need to monitor multiple systems when evaluating countermeasures to a given condition. The amino acid supplement did help maintain muscle mass (32), but it clearly had negative effects on bone. Ensuring the health of astronauts during spaceflight is critical and cannot be done successfully one system at a time. The findings from this study highlight that fact and emphasize the need for countermeasure validation and verification studies to account for multisystem effects.

The findings observed herein, that excess amino acids exacerbate bone resorption and calcium loss in an analog of weightlessness, are likely easily counteracted by addition of base precursors to the supplement or diet for subjects receiving the supplement. The findings are more important for the general public, especially for those consuming diets rich in protein and low in calcium or low in fruits and vegetables.

ACKNOWLEDGMENTS

The authors thank the Johnson Space Center Nutritional Biochemistry Laboratory team for support of this project. We also thank J. Krauhs and M. Heer for reviewing the manuscript.

GRANTS

This project was supported by the National Space Biomedical Research Institute (NPFR00205) through a cooperative agreement with National Aeronautics and Space Administration (CC 9-58).

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