Co-Administration of Proline and Inorganic Iron Enhance the Improvement of Behavioral and Hematological Function of Iron-Deficient Anemic Rats

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Summary We investigated the interrelationships between behavior and serum amino acid concentrations in iron-deficient anemic rats. Concentrations of proline, alanine, glycine, and phenylalanine in serum samples were significantly higher than those in rats fed a normal diet, while serum threonine, glutamic acid, and valine levels were significantly lower. Activities of locomotion, rearing, hole-poking, and grooming, determined by using a hole board apparatus, were significantly reduced in anemic rats. The supplementation of inorganic iron and amino acids proline, arginine, or glutamic acid to the normal diet lead to the recovery of normal behavior. Proline enhanced a significant increase in the number of red blood cells and hemoglobin by the supplementation of iron alone. We propose that the combination of amino acid (especially proline) and inorganic iron might lead to an improvement in behavioral disorders caused by iron-deficient anemia.

Key Words proline, arginine, glutamic acid, behavior, iron-deficient anemic rat

Plasma amino acid concentrations are, as a rule, stable in humans (1-4) and laboratory animals (5-8), with the exception of congenital amino acid metabolic disorders. However, drastic changes in the levels of amino acids in the plasma have been observed under conditions of surgical stress (9, 10), immobilization stress (11, 12), running stress (13, 14), and chronic fatigue syndrome (15). Thus, certain kinds of stress and disease can evoke changes in plasma amino acid concentrations.

Chvapil et al. reported that, in iron-deficient anemic rats, collagen protein formation was inhibited in granulation tissues and hypertrophic heart muscles; however, non-collagenous protein formation was significantly increased in both tissues (16). The specific activity of incorporated proline and the activity of formed hydroxyproline were significantly reduced under the condition of iron-deficient anemia. Furthermore, in anemic patients, the administration of iron led to increased excretion of urinary hydroxyproline, while this was not observed in patients in the control group (17, 18). These authors also indicated that iron might directly affect proline metabolism for the co-enzyme of proline hydroxylase. The effects of proline on iron-deficient anemic rats were not mentioned.

We attempted to clarify whether the simultaneous oral administration of proline or other amino acids with inorganic iron would lead to improvement in the symptoms of iron-deficient anemia.

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MATERIALS AND METHODS

Animals and experimental design. Weanling female Wistar rats (50-70 g; Japan SLC, Hamamatsu, Japan) were provided free access to a commercial non-purified diet (CE-2; Japan Clea, Inc., Tokyo, Japan) and tap water for 3 d. The animals were then divided into groups of six. The compositions of iron-deficient and normal diets used are shown in Table 1-1 and 1-2. The iron-deficient anemic rats were individually housed in stainless cages at a room temperature (25±2°C) and humidity (55%), with a 12-h light (7:00-19:00 h) and dark cycle. They were given free access to an iron-deficient diet and distilled water for 31 d. The animals were intragastrically administered proline (1 g/kg of body weight) and ferric citrate (iron. 1.5 mg/kg), arginine (1 g/kg) and iron (1.5 mg/kg), glutamic acid (1 g/kg) and iron (1.5 mg/kg), iron (1.5 mg/kg) alone, or only distilled water at 10:00 a.m. on the last 3 d in the experimental period. The experimental procedures used in this study met the guidelines of the Animal Care and Use Committee of the University of Shizuoka.

Behavioral analysis. Rats were carefully placed in the middle of a hole board apparatus, and the active motor behaviors were monitored via video for 10 min (19). The apparatus consisted of an opaque plastic area 70×70 cm in size, separated into 16 square fields of 17.5×17.5 cm, and surrounded by walls 50 cm high. A hole 2 cm in diameter was drilled into the squares adjacent to the walls, and the apparatus was elevated by 2 cm to allow for hole-poking. Four types of behaviors were assessed: locomotion (entering another field by at
Table 1-1. Composition of diet.

| Component          | Amount (%) | Normal  | Iron-deficient diet |
|--------------------|------------|---------|---------------------|
| α-Corn starch      | 42.57      | 42.57   |                     |
| Sucrose            | 21.28      | 21.28   |                     |
| Casein             | 20.0       | 20.0    |                     |
| Corn oil           | 5.0        | 5.0     |                     |
| Vitamin mix        | 1.0        | 1.0     |                     |
| Mineral mix        | 5.0        |         |                     |
| Mineral mix(-Fe)   | —          | 5.0     |                     |
| Cellulose          | 5.0        | 5.0     |                     |
| Choline-Cl         | 0.15       | 0.15    |                     |
| Total              | 100        | 100     |                     |

1 Purchased from Oriental Yeast Co., Tokyo, Japan.
2 Purchased from Honen Co., Tokyo, Japan.
3 Vitamin (AIN-93), mineral (AIN-93), and mineral(-Fe)
   (AIN-93) mixtures were purchased from Nihon Nosan
   K.K., Yokohama, Japan.
4 Purchased from Wako Pure Chemical Co., Osaka,
   Japan.

Table 1-2. Composition of the mineral mix, and mineral mix(-Fe) used for the normal and iron-deficient diets.

| Component          | Amount (mg/1000000 mg) | Normal  | Iron-deficient diet |
|--------------------|-------------------------|---------|---------------------|
| CaCO₃              | 3570000.00              | 3570000.00 |                     |
| KH₂PO₄             | 1960000.00              | 1960000.00 |                     |
| K₂C₆H₇O₆·H₂O       | 70780.00                | 70780.00   |                     |
| NaCl               | 74000.00                | 74000.00   |                     |
| K₂SO₄              | 46600.00                | 46600.00   |                     |
| MgO                | 24000.00                | 24000.00   |                     |
| FeC₆H₇O₅·nH₂O      | 6060.00                 |         |                     |
| ZnCO₃              | 1650.00                 | 1650.00   |                     |
| MnCO₃              | 630.00                  | 630.00    |                     |
| CuCO₃·Cu(OH)₂·H₂   | 300.00                  | 300.00    |                     |
| KIO₃               | 10.00                   | 10.00     |                     |
| Na₂SeO₄            | 10.25                   | 10.25     |                     |
| (NH₄)₆Mo₇O₂₄·4H₂O  | 7.95                    | 7.95      |                     |
| Na₂SiO₃·9H₂O       | 1450.00                 | 1450.00   |                     |
| Cr₂(SO₄)₃·12H₂O    | 275.00                  | 275.00    |                     |
| LiCl               | 17.40                   | 17.40     |                     |
| H₂BO₄              | 81.50                   | 81.50     |                     |
| NaF                | 63.50                   | 63.50     |                     |
| NiO₂·2Ni(OH)₂·2H₂O | 30.53                   | 30.53     |                     |
| NH₄VO₃             | 6.60                    | 6.60      |                     |
| Sucrose            | up to 1000000           | up to 1000000 |                     |

Biochemical analyses. The rats were anesthetized with pentobarbital at 10:00 a.m. after a 16 h fast on the last day of the experiment period, and blood samples were withdrawn from the abdominal vein into plastic syringes. Ethylenediamine-tetraacetic acid dipotassium salt was used as the anticoagulant. Tissues were immediately removed, frozen on dry ice and stored at −80°C until needed for assay. The serum iron and unsaturated iron-binding capacity were enzymatically measured using commercial kits (Fe C and UIBC Test Wako, respectively; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Blood tests (counts of red blood cells, leukocytes, and platelets, and hemoglobin and hematocrit values) were done using an automatic analyzer (Technicon H·1E; Hitachi Co. Ltd., Tokyo, Japan). Concentrations of serum amino acids were determined using an automatic amino acid analyzer (L-8500; Hitachi Co. Ltd.).

Statistics. The statistical significance of the differences between values was determined using an analysis of variance and Duncan’s multiple-range test (20).

RESULTS

The effects of ingesting an iron-deficient diet on mean daily growth, food intake, and organ weights of rats are shown in Table 2. The body weights of rats fed the iron-deficient diet were significantly lower than those of the rats fed a normal diet for 8, 16 and 31 d (p<0.05). Food intake and liver weights of rats fed the iron-deficient diet were significantly lower than those of rats fed the normal diet for 16 and 31 d (p<0.05).

A hematogram of rats fed the iron-deficient diet is shown in Fig. 1. The numbers of red blood cells, hematocrit value frequencies and hemoglobin levels of rats fed the iron-deficient diet were significantly lower than those of rats fed the normal diet for 8, 16, and 31 d (p<0.05). The numbers of leukocytes and platelets of rats fed the iron-deficient diet were significantly higher than those of rats fed the normal diet (p<0.05). Thus, rats fed the iron-deficient diet had induced anemia, and growth and body weight were reduced.

Certain stresses and diseases evoke changes in serum amino acid concentrations (5, 21–23). In the anemic rats, the concentrations of serum proline, alanine, glycine, and phenylalanine were increased, and those of serum threonine, glutamic acid, and valine were decreased. Therefore, serum amino acid concentrations were also altered in the iron-deficient anemic rats (Table 3).

To determine if these changes in the anemic rats would affect behavioral activities, we used a hole board apparatus and videotaped the activities of the rats. In the first study, gavage of proline, which increased the levels of serum amino acids in anemic rats, glutamic acid, which was decreased, and arginine, which was unaltered, were executed. In every case, hematological parameters and behavioral activities were unchanged in the iron-deficient anemic rats (Table 4). Since it is generally impossible to remove iron from daily foods, we questioned if the simultaneous oral administration of proline, glutamic acid, and arginine and

least 3/4 of the body), rearing on the hind legs, hole-poking, and grooming. Each behavioral activity was monitored and recorded on video, and the tapes were subsequently shown to an observer blinded as to the experimental conditions. Independent behavioral rating scores for each rat were thus obtained.
Proline and Iron Deficient Anemia  

Table 2. Effect of an iron deficient diet on growth, food intake, and organ weight of rats for 0, 8, 16 and 31 d. 

| Measurement | Normal | Iron-deficient diet |
|-------------|--------|---------------------|
| Days        | 0      | 8                   |
|             | 16     | 31                  |
| Body weight (g) | 52.0±1.70 | 85.9±0.99           |
| Body weight gain (g) | 114.6±1.75 | 143.80±2.38       |
| Food intake (g) | 63.0±0.177 | 109.05±2.36        |
| Organ weight (g/100 g BW) | 2.66±0.07 | 1.73±0.03          |
| Brain        | 1.37±0.03 | 1.11±0.01           |
| Liver        | 4.67±0.06 | 4.08±0.06           |
| Kidney       | 0.84±0.015 | 0.761±0.017        |
| Adrenal glands | 0.0252±0.0009 | 0.0233±0.0006    |
| Spleen       | 0.313±0.013 | 0.238±0.034        |

Each value is expressed as the mean±SE of 6 rats per group. Different letters represent significant differences (p<0.05).

Table 3. Serum amino acid concentrations in rats fed normal and iron-deficient diets for 31 d. 

| Amino acids (nmol/mL) | Normal | Iron-deficient diet |
|-----------------------|--------|---------------------|
| Proline               | 537.0±81.3 | 1064.4±37.8        |
| Alanine               | 558.4±77.9 | 1056.7±37.6        |
| Glycine               | 173.0±9.6 | 257.3±9.0          |
| Histidine             | 104.3±14.2 | 138.0±9.4          |
| Phenylalanine         | 101.6±9.3 | 125.5±1.6          |
| Lysine                | 869.6±79.8 | 919.5±25.2        |
| Methionine            | 91.9±8.9 | 97.0±1.4           |
| Serine                | 303.8±22.0 | 311.9±6.4         |
| Tyrosine              | 158.9±22.0 | 151.6±6.7         |
| Arginine              | 210.2±23.8 | 200.2±4.1         |
| Urea                  | 9362.8±454 | 8797.5±285        |
| Tryptophan            | 192.3±16.2 | 180.3±1.6         |
| Isoleucine            | 89.0±7.8 | 79.1±1.7           |
| Threonine             | 234.2±20.3 | 204.0±11.8        |
| Leucine               | 257.9±20.8 | 210.9±5.7         |
| Cysteine              | 24.7±2.5 | 20.0±1.0          |
| Valine                | 375.6±27.7 | 299.0±8.0         |
| Aspartic acid         | 31.5±2.7 | 25.0±2.2          |
| Glutamic acid         | 226.9±14.6 | 157.9±7.1         |
| Threonine             | 523.1±42.7 | 246.7±9.3         |

Each value is expressed as the mean±SE of 6 rats per group. Different letters represent significant differences (p<0.05).

inorganic iron (dosage equivalent to approximately 1/3 daily iron intake in rats) would alter the hematogram and behavioral activity in iron-deficient anemic rats.

These behavioral activities are shown in Table 4. As for the iron-deficient anemic rats, all action activities were significantly lower than those of normal rats. As for the rearing behavior, the iron alone and combined amino acid and iron ingesting iron-deficient anemic rats displayed a tendency toward improvement as compared to the control group.

The locomotive activities of anemic rats showed improvement when iron was given alone. Also, the simultaneous administration of proline or glutamic acid resulted in a significant increase in locomotion activity.

The hole-poking behavior of the iron-deficient anemic rats progressed towards recovery after the administration of proline and iron.

There was a significant improvement in grooming behavior following the administration of a combination of each amino acid and iron. The group administered the proline and iron combination showed a value almost equivalent to the normal group.

These behavioral results clearly indicated that the co-administration of inorganic iron and amino acids, especially proline, is effective for improving behavioral disorders evoked by iron-deficient anemia.

In anemic rats, the counts of red blood cells and hemoglobin were significantly decreased (Fig. 2a). The administration of iron improved the numbers, and the co-administration of proline further improved the red
blood cell count. The hemoglobin content increased significantly following the ingestion of proline and iron, as compared to other groups (Fig. 2b). Thus, the behavioral improvement seen in the iron-deficient anemic rat could underlie these hematological effects upon the co-administration of iron and proline. The numbers of leukocytes and platelets, hematocrit value, serum iron, and unsaturated iron-binding capacity were unaltered in each group (data is not shown).

**DISCUSSION**

In this study, we clearly observed that proline, which was increased in the serum concentration of iron-deficient anemic rats as opposed to the normal rats, brought on an ameliorative effect of iron-deficient anemia when co-administered with iron to iron-deficient anemic rats. Here, we discuss the possible mechanism underlying the increase of serum proline concentration and the amelioration effect of proline.

**Potential mechanisms underlying the increase of serum proline concentration**

It is known that there are intracellular and/or extracellular amino acid pools in the liver (24), muscle (25) and brain (26). It is conceivable that these amino acid pools play an important part in the supply of proline into the blood. Actually, according to our recent data, water-immersion stress increases the branched chain amino acid concentration in rat serum, mitigating the stress reaction provoked by water-immersion stress (27). In addition, in a human study, subjects with fatigue had high levels of certain kinds of serum gluconeogenic amino acids (28). The authors of this paper...
have suggested that these alter the level of serum free-amino acids that seem to increase the secretion of adrenal corticoid hormone caused by the stress of fatigue (28). Thus, these previous studies suggested that the concentration of humoral free-amino acids in the body readily change in a stress-specific manner, suggesting a physiological function designed to accomplish an end.

Actually, there is biochemical evidence of the importance of proline to the structural and functional futures of iron-binding proteins. It is known that proline is a major product of the hydrazinolysis of transferrin (29). In addition, Takagi et al. (30) demonstrated that the proline-containing iron entry site of the H-subunit of ferritin plays an important role in regulating iron release. Moreover, in a recent study, heme oxygenase-2 (31) was shown to have two copies of the heme regulatory motif with a conserved core that includes two proline residues.

From these previous reports, the present results could be interpreted to suggest that the increase in serum proline concentration might be due to supplementation from amino acid pools. It is an increase in serum proline concentration that is necessary for the synthesis of iron related proteins such as ferritin, transferrin, and possibly hemoglobin, which are changed with iron-deficient anemia (32). This explanation could also describe why only the co-administration of proline works to improve iron-deficient anemia.

In addition, Chvapil et al. reported that collagen protein formation was inhibited in iron-deficient anemic rats because of the low activity of proline hydroxylase, which requires iron and ascorbic acid as cofactors (16). This biochemical phenomenon also could be an underlying factor for the increase in serum proline concentration.

Improvement in behavior of iron-deficient anemic rats given dietary iron and proline

In this study, we attempted to clarify whether or not the co-administration of inorganic iron and amino acids is effective for improving behavioral disorders in iron-deficient anemic rats. The present results clearly demonstrate that the co-administration of proline and inorganic iron to iron-deficient anemic rats led to an improvement in behavioral disorders, while arginine and glutamic acid did not.

From the hematological data, we propose that the improvement effect of proline on behavioral studies is attributable to improving the oxygen supply to peripheral cells, which are dependent on increased red blood cell count and hemoglobin levels.

This behavioral improvement data also could be interpreted to suggest that the co-administration of proline and iron directly affects the emotional activity of iron-deficient anemic rats. At present, however, we have no concrete evidence to support this hypothesis.

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