Dietary Copper Deficiency Reduces Iron Absorption and Duodenal Enterocyte Hephaestin Protein in Male and Female Rats

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ABSTRACT The mechanism for reduced Fe absorption in Cu deficiency is unknown, but may involve the intestinal Cu-dependent ferroxidase, Hephaestin (Hp), a 2-electron Cu oxidase; WBC, whole-body-counting.

Copper (Cu) is required for the efficient utilization of iron (Fe) in mammalian systems (1). Signs of Fe deficiency anemia appear quickly in rats fed a Cu-deficient diet, and include low blood hemoglobin (Hgb), low hematocrit (Hct), and low RBC count (2–4). Earlier studies in the 1950s and 1960s showed that Cu deficiency reduced Fe absorption in pigs (5,6) and rats (7). However, the diets used in those studies were unusual by today’s standards, and consisted of condensed whole cow’s milk diluted 1:1 with water, and supplemented with Fe and Cu. Later, more nutritionally complete diets were used to study the effects of Cu deficiency on Fe absorption. Unfortunately, some of the findings were rather ambiguous compared with those in the earlier work. Cohen et al. (8) found anemia and low ceruloplasmin (Cp) activity in both Cu-deficient male and female offspring of Cu-deficient dams, but only deficient females had reduced Fe absorption. Copper and Davies (9) showed that Cu deficiency in rats fed a purified diet did not affect Fe uptake into enterocytes from the gut lumen, but Fe transfer to the blood stream was reduced by 50%. Failla and Seidel (10) measured total body Fe in Cu-deficient and control rats, and found no differences between the groups, suggesting that Fe absorption over the course of the experiment was not reduced even though Hct was reduced by as much as 30% in the Cu-deficient groups. Later, Thomas and Oates (11) presented evidence that Fe absorption was actually enhanced in Cu-deficient female rats. To find a clearer answer to this question, Reeves and DeMars (3) used radioactive 59Fe and the whole-body counting (WBC) technique (12) to determine the effects of Cu deficiency on Fe absorption. They showed that Cu-deficient male rats absorbed less Fe than Cu-adequate rats and were severely anemic (3). Although it was observed that male and female rats respond differently to Cu deficiency (8,13,14), there is a need to determine whether Fe absorption differs between the sexes.

The mechanisms that explain reduced Fe absorption in Cu deficiency are unknown, but might involve changes in some of the Cu-dependent proteins in the duodenal enterocyte involved in regulating Fe efflux across the basolateral membrane into the circulation. One candidate is Hephaestin (Hp), a multi-Cu ferroxidase expressed in the intestinal enterocytes and similar to Cp in liver and plasma (15). The Fe efflux transporter, ferroportin1 (FPN1), located in the basolateral membrane, is involved in the Fe efflux from the enterocyte. Cu deficiency reduces Fe absorption in rats through reduced expression of duodenal Hp protein.

KEY WORDS: • copper deficiency • iron • absorption • biological half-life • rats
membrane of the enterocyte, transports Fe only in the oxidized state (16); this oxidation step may be carried out by Hp. Indeed, the sla mouse, which contains an Hp protein with a reduced efficiency to oxidize Fe\(^{2+}\), cannot transport Fe and becomes Fe deficient (15,17,18). The activity of Cu-dependent Cp is severely reduced in serum and liver of Cu-deficient rats; thus, it is logical to conclude that the activity of the Cu-enzyme Hp would be affected similarly. Han and Wessling-Resnick (19) observed that Hp mRNA was lower in Caco-2 cells (enterocyte mimics) not treated with 1 \(\mu\)mol Cu/L of medium than those that were. Chen et al. (personal communication)\(^5\) reported that both Hp protein and oxidase activity were reduced in CuD HT29 cells, another enterocyte mimic. Work by Nittis and Gitlin (20) on the role of Cu in the cellular degradation of Hp suggests a link between enterocyte Fe regulation and nutritional Cu status. However, to date, Hp protein and oxidase activity have not been reported in Cu-deficient animals. Thus, our goal was to determine the effect of Cu deficiency on Fe absorption and metabolism between male and female rats, and to evaluate the effects of Cu deficiency on the relative amount of Hp protein in the duodenal enterocytes. We also assessed the effects of sex on the signs of Cu deficiency, severity of anemia, and mineral composition of blood and various organs.

**MATERIALS AND METHODS**

These studies were approved by the Animal Use Committee of the USDA-ARS, Grand Forks Human Nutrition Research Center. The procedures followed the NIH guidelines for the experimental use of laboratory animals (21).

**Animals and treatment.** The study design was a 2 × 2 factorial and consisted of 2 treatment groups, Cu-adequate (CuA) and Cu-deficient (CuD) with both male and female rats. The diets were based on the AIN-93G formulation (22,23) and contained 5.0 mg Cu/kg in the CuA group and 0.25 mg Cu/kg in the CuD group. This diet contained Fe in the form of ferric citrate. At 3 wk of age, each group of 16 male and 16 female Sprague-Dawley rats [Strain: SAS/VAF (SD)] was randomly divided into 2 groups of 8 rats each and fed either of the 2 diets. Rats were housed in a temperature-controlled room at 22°C with 50% humidity, and a light (0600 –1800 h):dark (1800 –0600 h) cycle. Body weights were recorded weekly.

**Whole-body and fecal counting.** After consuming the experimental diet for 19 d, each rat was deprived of food from 0800 to 1800 h and prepared for the determination of Fe absorption using WBC to measure \(^{59}\)Fe absorbed from the diet. These techniques were identical to those outlined earlier by Reeves and DeMars (3). To determine the effect of Cu deficiency on the excretion patterns of \(^{59}\)Fe, feces were collected daily from each rat and analyzed for \(^{59}\)Fe using a Packard Cobra gamma counter. Beginning 5 cm from the pylorus, 5 cm of the duodenum was removed and the lumen was washed with 10 mL of cold saline. The segment was slit open and the mucosal layer gently scraped off with the edge of a glass slide. The liver, but not the kidney or spleen, was perfused with cold saline to remove Fe. A 1.0-g piece of perfused liver was assayed for \(^{59}\)Fe and then lyophilized and digested with nitric acid for the determination of Fe and Cu by inductively coupled argon plasma spectroscopy. One whole kidney, whole spleen, and mucosal scrapings were analyzed similarly for \(^{59}\)Fe, Fe, and Cu content.

**Statistical analysis.** For each rat, log-linear regression analyses of the percentage of isotope retention vs. time in days were conducted on the linear part of the curves between d 6 and 12. The y-intercept from each regression was used to estimate the percentage absorption of \(^{59}\)Fe. The biological half-life (BHL) of \(^{59}\)Fe was calculated as \(-\ln (2)/\text{slope} ). Because the distributions of the BHL were skewed, these data were In-transformed before statistical analyses were performed. Statistical differences between groups for absorption and BHL were tested by 2-way ANOVA. Two-way ANOVA also was used for all other variables. Differences were considered significant at \(P \leq 0.05\). When interactions were significant, Tukey’s contrasts were used to identify differences between means.

**RESULTS**

Both male and female rats fed the CuD diet developed signs of Cu deficiency. CuD rats gained less weight than CuA rats,
Effects of Cu deficiency on weight gain, serum Cu and Fe, and serum and muscle Cu-dependent enzyme activities in male and female rats

### TABLE 1

<table>
<thead>
<tr>
<th>Sex</th>
<th>Treatment</th>
<th>Weight gain, g/d</th>
<th>Serum Cu μmol/L</th>
<th>Serum Fe μmol/L</th>
<th>Serum CpAO activity U/L</th>
<th>Serum SOD3 activity U/L</th>
<th>Soleus muscle CO1 activity nmol/(min - mg protein)</th>
<th>Gastrocnemius muscle CO1 activity nmol/(min - mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>CuA</td>
<td>6.9 ± 0.2</td>
<td>13.20 ± 0.65</td>
<td>52.6 ± 3.9</td>
<td>86.7 (83.6–89.8)</td>
<td>136 ± 6</td>
<td>244 ± 2 (&lt;0.001)</td>
<td>380 (320–450)</td>
</tr>
<tr>
<td>M</td>
<td>CuD</td>
<td>6.5 ± 0.2</td>
<td>0.60 ± 0.04</td>
<td>30.0 ± 2.4</td>
<td>0.24 (0.19–0.30)</td>
<td>25 ± 4</td>
<td>75 ± 11&lt;0.001</td>
<td>67 (59–76)</td>
</tr>
<tr>
<td>F</td>
<td>CuA</td>
<td>5.4 ± 0.2</td>
<td>14.73 ± 0.73</td>
<td>56.3 ± 4.4</td>
<td>112.8 (108.5–117.3)</td>
<td>147 ± 5</td>
<td>189 ± 7b &lt;0.001</td>
<td>357 (343–372)</td>
</tr>
<tr>
<td>F</td>
<td>CuD</td>
<td>4.8 ± 0.2</td>
<td>0.81 ± 0.13</td>
<td>29.5 ± 4.4</td>
<td>0.17 (0.11–0.26)</td>
<td>26 ± 1</td>
<td>96 ± 7c &lt;0.001</td>
<td>112 (100–124)</td>
</tr>
</tbody>
</table>

Statistical comparisons

- **P-values**
  - **Sex**: NS
  - **Treatment**: NS
  - **S × T**: NS

### TABLE 2

Effects of Cu deficiency on the blood chemistry of male and female rats

<table>
<thead>
<tr>
<th>Sex</th>
<th>Treatment</th>
<th>Hgb g/L</th>
<th>Hct 10⁶/μL</th>
<th>RBC</th>
<th>MCV</th>
<th>MCHC</th>
<th>RDW</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>CuA</td>
<td>132 ± 1⁠&lt;a&gt;</td>
<td>0.40 ± 0.04&lt;⁠&lt;a&gt;</td>
<td>7.17 ± 0.11&lt;⁠&lt;a&gt;</td>
<td>54.0 ± 1.9</td>
<td>331 ± 2</td>
<td>15.1 ± 0.3</td>
</tr>
<tr>
<td>M</td>
<td>CuD</td>
<td>85 ± 5&lt;⁠&lt;c&gt;</td>
<td>0.27 ± 0.02&lt;⁠&lt;c&gt;</td>
<td>5.69 ± 0.35&lt;⁠&lt;b&gt;</td>
<td>47.7 ± 1.3</td>
<td>315 ± 3</td>
<td>26.6 ± 1.5</td>
</tr>
<tr>
<td>F</td>
<td>CuA</td>
<td>136 ± 2&lt;⁠&lt;a&gt;</td>
<td>0.40 ± 0.05&lt;⁠&lt;a&gt;</td>
<td>7.48 ± 0.21&lt;⁠&lt;a&gt;</td>
<td>54.7 ± 1.9</td>
<td>333 ± 2</td>
<td>14.9 ± 0.5</td>
</tr>
<tr>
<td>F</td>
<td>CuD</td>
<td>104 ± 5&lt;⁠&lt;b&gt;</td>
<td>0.33 ± 0.12&lt;⁠&lt;b&gt;</td>
<td>7.34 ± 0.37&lt;⁠&lt;b&gt;</td>
<td>44.4 ± 1.0</td>
<td>319 ± 3</td>
<td>27.1 ± 1.8</td>
</tr>
</tbody>
</table>

Statistical comparisons

- **P-values**
  - **Sex**: NS
  - **Treatment**: NS
  - **S × T**: NS

### Notes

1. Values are means ± SEM, n = 8. Means in a column without a common letter differ, P < 0.05.
2. Not significant, P > 0.05.
various organs of rats. The distribution of 59Fe in whole blood, expressed as a percentage of the WBC on the final day of the experiment, was similar to the pattern of Hgb concentration among groups (Tables 2 and 3). There was ~17% less (P < 0.001) 59Fe in the blood of CuD rats than in CuA rats, with no effect of sex on this variable. The percentage of total body 59Fe in the liver of CuD male rats was 4 times (P < 0.001) that of CuA rats and doubled in the livers of CuD female rats compared with CuA rats; however, females had more label in their livers than males (P < 0.001). On the other hand, kidney 59Fe was lower (P < 0.001) in female rats, but only if they were also Cu deficient (Table 3). More 59Fe was distributed to the spleen in CuD rats than CuA rats, and females had less of the label than males (Table 3).

Copper concentrations in all organs were lower in CuD rats than CuA rats (Table 4). Cu deficiency also affected the amount of nonradioactive Fe in various organs (Table 4). The concentration of Fe in the duodenal mucosa was higher (P < 0.014) in CuD rats compared with the controls (Table 4). Overall, female rats had higher (P < 0.001) liver Fe than males (Table 4). Also, the liver of male CuD rats contained more Fe than CuA rats (P < 0.005). There was an interaction (P < 0.001) between treatment and sex for kidney Fe showing that Cu deficiency lowered kidney Fe more in female than in male rats. The concentration of Fe in the spleen of CuD rats was lower (P < 0.001) than that in CuA rats.

If Cu deficiency truly reduces the amount of dietary Fe absorbed, then the Fe concentration in the whole body should be less than that in CuA rats. The total amounts of Fe contributed by liver, kidneys, spleen, and total blood were summed and expressed as μmol/100 g body weight. The values were (mean ± SEM, n = 8): Male-CuA, 61.7 ± 0.8; Male-CuD, 43.2 ± 1.9; Female-CuA, 71.2 ± 1.0; Female-CuD, 59.0 ± 2.7. Body Fe concentration was significantly (P < 0.001) less in CuD rats than in CuA rats, and males had less (P < 0.001) body Fe than females.

DISCUSSION

Recently, our laboratory reported that male rats fed CuD diets for at least 25 d absorbed significantly less Fe than CuA rats (3). Yet, shortly before that report was published, Thomas and Oates (11) found that Fe absorption was actually enhanced in CuD female rats. Because their procedures differed from ours, we decided to compare Fe absorption in CuD and CuA male and female rats using our standard WBC technique.
This involved feeding rats a single $^{59}$Fe-labeled meal of their respective diets and measuring the amount of $^{59}$Fe consumed, and then measuring its disappearance over a period of 12 d. Extrapolation of the log-linear portion of the decay curve back to zero time gives an estimate of the amount of $^{59}$Fe initially absorbed. The results clearly showed that feeding the CuD diets for only 19 d lowered intestinal Fe absorption in both male and female rats.

A mechanism for reduced Fe absorption in Cu deficiency may lie in its effect on Hp protein and activity. Chen et al. (5) showed that Hp protein and ferroxidase activity were reduced in rapid loss of Hp protein. Results presented in the current study support those findings. Western blotting showed that the relative amount of Hp protein in isolated duodenal enterocytes of CuD rats was reduced to 30% of that in CuA rats. Thus, the reduction in the relative amount of the multi-Cu Hp protein in the duodenum is directly correlated with the rate of Fe absorption. We propose that this is at least part of the cause of Fe deficiency in CuD rats.

However, because CuD-induced anemia is not cured in some animal models by injecting them with Fe, there must be other factors involved (5). In a temporal study, we found that young rats deprived of dietary Cu for only 7 d were beginning to experience reduced Fe absorption compared with CuA rats, but the difference was not significant (3). Nonetheless, signs of Cu deficiency such as very low serum Cu and serum CpAO activity were already evident at d 4. In the same experiment, signs of anemia were not observed until between d 7 and 14, which is reasonable, because the half-life of rat erythrocytes is ~14 d (31).

Systemic administration of Fe did not cure anemia in CuD swine (5), supporting that Cu has an essential role in blood cell formation and/or heme synthesis. Indeed, erythrocytes are smaller and the maturation of neutrophils and granulocytes is impaired in Cu deficiency (32–34). Williams et al. (35) also showed that heme synthesis was reduced in liver mitochondria of both CuD pigs and rats (36), suggesting that similar effects would be seen also in bone marrow. However, Lee et al. (37) reported earlier that Cu deficiency had no effect on heme synthesis in whole-blood reticulocytes of swine, and concluded

### TABLE 3

<table>
<thead>
<tr>
<th>Sex</th>
<th>Treatment</th>
<th>Total blood(^2)</th>
<th>Total liver</th>
<th>Total kidneys</th>
<th>Total spleen</th>
<th>% final WBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>CuA</td>
<td>41.4 ± 0.9</td>
<td>2.0 ± 0.2</td>
<td>0.41 ± 0.02(^{b})</td>
<td>0.77 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>CuD</td>
<td>34.5 ± 1.6</td>
<td>8.1 ± 0.9</td>
<td>0.54 ± 0.03(^{a})</td>
<td>1.07 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>CuA</td>
<td>40.8 ± 1.4</td>
<td>7.4 ± 0.9</td>
<td>0.41 ± 0.02(^{b})</td>
<td>0.61 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>CuD</td>
<td>30.0 ± 2.4</td>
<td>15.6 ± 1.4</td>
<td>0.35 ± 0.03(^{b})</td>
<td>0.76 ± 0.05</td>
<td></td>
</tr>
</tbody>
</table>

Statistical comparisons

<table>
<thead>
<tr>
<th>Sex</th>
<th>Treatment</th>
<th>(P)-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Values are means ± SEM, \(n = 8\). Means in a column without a common letter differ, \(P < 0.01\).

\(^2\) Total blood volume was calculated based on the assumption that it represents 7% of the rat’s body weight.

\(^{a}\) Not significant, \(P > 0.05\).

### TABLE 4

<table>
<thead>
<tr>
<th>Sex</th>
<th>Treatment</th>
<th>Duodenal mucosa Cu</th>
<th>Duodenal mucosa Fe</th>
<th>Liver Cu</th>
<th>Liver Fe</th>
<th>Kidney Cu</th>
<th>Kidney Fe</th>
<th>Spleen Cu</th>
<th>Spleen Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(\mu)mol/kg</td>
<td>(\mu)mol/kg</td>
<td>(\mu)mol/kg</td>
<td>(\mu)mol/kg</td>
<td>(\mu)mol/kg</td>
<td>(\mu)mol/kg</td>
<td>(\mu)mol/kg</td>
<td>(\mu)mol/kg</td>
</tr>
<tr>
<td>M</td>
<td>CuA</td>
<td>125.5 ± 4.6</td>
<td>2.70 ± 0.40</td>
<td>165.4 ± 4.5</td>
<td>2.38 (2.29–2.48)(^{c})</td>
<td>408 ± 17</td>
<td>2.57 ± 0.15(^{b})</td>
<td>76.4 ± 2.0</td>
<td>19.7 ± 1.1</td>
</tr>
<tr>
<td>M</td>
<td>CuD</td>
<td>47.5 ± 6.2</td>
<td>4.27 ± 0.49</td>
<td>20.3 ± 4.4</td>
<td>3.71 (3.48–3.96)(^{b})</td>
<td>178 ± 6</td>
<td>2.33 ± 0.05(^{b})</td>
<td>12.7 ± 1.8</td>
<td>14.3 ± 0.9</td>
</tr>
<tr>
<td>F</td>
<td>CuA</td>
<td>129.4 ± 1.9</td>
<td>3.38 ± 0.16</td>
<td>179.0 ± 7.9</td>
<td>9.26 (8.59–9.98)(^{a})</td>
<td>522 ± 57</td>
<td>3.32 ± 0.04(^{a})</td>
<td>75.2 ± 2.0</td>
<td>19.0 ± 0.9</td>
</tr>
<tr>
<td>F</td>
<td>CuD</td>
<td>32.2 ± 1.7</td>
<td>4.63 ± 0.84</td>
<td>22.9 ± 1.8</td>
<td>8.83 (9.01–10.49)(^{a})</td>
<td>177 ± 5</td>
<td>2.35 ± 0.09bc</td>
<td>12.9 ± 2.2</td>
<td>14.0 ± 0.5</td>
</tr>
</tbody>
</table>

Statistical comparisons

<table>
<thead>
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<th>(P)-values</th>
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</tbody>
</table>

\(^1\) Values are means ± SEM, \(n = 8\). Because the distributions of values for liver Fe were skewed, these data were ln-transformed before statistical analyses were performed. The tabulated values represent the back transformation of the means with the SEM range. Means in a column without a common letter differ, \(P < 0.01\).

\(^2\) Not significant, \(P > 0.05\).
that globin synthesis might be impaired. Apparently, no one has examined the effects of Cu deficiency on heme or globin synthesis in the bone marrow. Even so, at least one report counters this argument by showing that mice recover from the CuD-induced anemia when given subcutaneous injections of Fe (38).

In the current study, the severity of Cu deficiency did not differ between males and females as substantiated by similar changes in tissue Cu concentrations and serum CpAO and SOD3 activities. Both sexes showed signs of CuD-induced Fe deficiency by reductions in numerous blood variables usually seen in Fe-deficient rats, including low blood Hgb, Hct, and serum Fe. However, these signs were more pronounced in CuD males than females, and RBC count was reduced in males but not females. Reasons for the lower susceptibility of females to Cu deficiency-induced anemia are not clear, but mechanisms directing the available Fe to erythrocyte maturation and heme synthesis might be less impaired in females than males.

Cu-deficient rats tend to accumulate excess Fe in their livers (4), and the findings in the current study were as expected when male rats were used. However, when liver Fe was measured in female rats, CuD and CuA rats did not differ. On the other hand, CuD female rats distributed twice as much of the body-$^{59}$Fe to the liver as the CuA rats. It also was shown that liver Fe is not elevated in CuD swine (5). The current theory is that the ferroxidase component of Cp serves to oxidize Fe$^{2+}$ to Fe$^{3+}$, thus enabling Fe transport out of the liver by FPN1. CpAO activities in female rats of the current study, and in the pigs mentioned above, were very low, but liver Fe remained normal. It was suggested by some investigators that CpAO activity of only 1% is sufficient to stimulate Fe efflux from cells to the circulation (39). CpAO activities of CuD female rats in the current study were only 0.15% of the CuA rats, but there was no difference in liver Fe. We found earlier (3) that after 4 d of Cu deficiency, serum CpAO activities in male rats, but there was no difference in liver Fe. We found that liver Fe of CuD male rats was reduced to 3% of controls, but there was no change in liver Fe. However, after 7 d of deficiency, CpAO activity was reduced to 1% of the controls in CuD rats, and liver Fe was elevated by 50% ($P < 0.001$). Harris et al. (40) found that liver Fe concentrations were higher in mutant mice with no functional Cp gene than in wild-type mice. They also showed that aceruloplasminemic mice had higher spleen iron than wild-type mice; however, as shown in the present study, spleen Fe in CuD rats was actually lower than in CuA rats. Thus, the role of Cp in regulating liver and spleen Fe concentration in Cu deficiency still has to be resolved.

In this study and a previous one (3), the turnover rate of body Fe was higher in CuD rats than in CuA rats. The reason for this is not known at present; however, Bush et al. (41) calculated that the turnover rate of systemic iron in CuD pigs was 5 times higher than that in CuA pigs. More than likely, Fe is being lost through secretions into the gut and/or sloughing of the enterocytes. We found previously that the loss of an oral dose of $^{59}$Fe was 3 times higher in the feces of CuD rats than CuA rats (3).

In summary, the principal finding of this study was that Cu deficiency decreased intestinal Fe absorption in both male and female rats. The decreased iron absorption caused by Cu deficiency seems to be consistent with a reduction in the relative protein concentration of the Cu-dependent intestinal ferroxidase, Hp. This causes more Fe in the enterocyte to remain in a reduced state, which in turn cannot be transported across the basolateral membrane by FPN1. Even though total-body Fe is reduced by Cu deficiency, it is possible that feedback signals for stimulating Fe absorption also are impaired by Cu deficiency. Further research is warranted to determine the exact mechanism through which Cu helps regulate intestinal Fe absorption.

ACKNOWLEDGMENTS

We acknowledge the assistance of Denice Schafer and staff for care of the animals; Jim Lindlauf for preparation of the diets; Brenda Skinner for assistance with animal kills and blood analyses on the Cell-Dyn; Kim Michelsen for the serum SOD3 and muscle CO1 analyses; Susan Fleck-Shepard and Deb Johnson for mineral analyses; and LuAnn Johnson for the statistical analyses.

LITERATURE CITED


