Quantifying Manganese in Lymphocytes to Assess Manganese Nutritional Status

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To clarify whether manganese nutritional status is better reflected by the manganese concentration in lymphocytes or in whole blood, we injected manganese solutions intravenously into manganese-deficient rats and determined manganese concentrations in lymphocytes, whole blood, and various tissues. The manganese concentrations in lymphocytes and tissues, but not in whole blood, were significantly less in manganese-deficient rats than in normal rats. These low values could be prevented by intravenous injection of manganese in a dose-dependent manner. These results suggest that, for assessment of manganese nutritional status, measurement of manganese in lymphocytes is better than that in whole blood.

Additional Keyphrases: manganese-deficient rats • atomic absorption spectrophotometry • parenteral nutrition

The technique of total parenteral nutrition (TPN) was introduced by Dudrick et al. (1) in 1968. The recent improvements in patient management and in the composition of TPN solutions have made it more likely that such nutrition is adequate to maintain the life of the patient. With an increasing number of patients depending solely on TPN, the necessity for supplementation with essential trace elements has been recognized (2–4).

Manganese is one of the essential trace elements that should be supplemented during TPN (5). Over- or underdosage of manganese can cause metabolic complications (6, 7), making it important to assess the nutritional status of manganese during TPN. We have previously reported that manganese concentrations in whole blood or plasma do not correlate to that in various tissues during TPN in rats (8). Here we report a study to clarify whether manganese concentration in lymphocytes reflects the manganese nutritional status. We measured manganese concentrations in lymphocytes and various tissues from manganese-deficient or -sufficient rats.

Materials and Methods

Apparatus

For analysis of manganese, we used a flameless atomic absorption spectrophotometer with a graphite furnace (AA-646; Shimadzu Co., Ltd., Kyoto, Japan) or a flame atomic absorption spectrophotometer (AA-670; Shimadzu Co., Ltd.). For lymphocytes count, we used an automated blood-cell counter (cc-108; Toa Iyou Denshi Co., Ltd., Hyogo, Japan). For ashing, we used a heating-block (TPB-62; Advantec Toyo Inc., Tokyo, Japan). Acid-washed glassware, polyethylene Pasteur pipettes, and polypropylene tubes were used throughout the study.

Table 1. Composition of Synthetic Diets

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Min-free</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Corn starch</td>
<td>37.0000</td>
<td>37.0000</td>
</tr>
<tr>
<td>Milk casein</td>
<td>25.0000</td>
<td>25.0000</td>
</tr>
<tr>
<td>α-Potato starch</td>
<td>10.0000</td>
<td>10.0000</td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>8.0000</td>
<td>8.0000</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>6.0000</td>
<td>6.0000</td>
</tr>
<tr>
<td>Salt mixture</td>
<td>8.0000</td>
<td>8.0000</td>
</tr>
<tr>
<td>Granulated sugar</td>
<td>5.0000</td>
<td>4.9884</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>1.0000</td>
<td>1.0000</td>
</tr>
<tr>
<td>MnCO3</td>
<td>0.10</td>
<td>11.60</td>
</tr>
</tbody>
</table>

* Mn-free salt mixture contained (mg/100 g diet): CaHPO4, 3385; CaCO3, 425; NaCl, 860; MgO, 417; K2(C2H3O2)2; H2O, 1873; K2SO4, 449; Fe citrate, 72.0; AI2(SO4)3; 12H2O, 43.2; CuCO3, 1.4; ZnCO3, 9.04; CoCl2, 0.152; KIO3, 0.776; granulated sugar, 884.432.

b Vitamin mixture contained (mg/100 g diet): vitamin B1, 0.6; vitamin B2, 0.6; vitamin B6, 0.7; vitamin B12, 0.005; vitamin A (400 int. units); vitamin E, 5.0; vitamin D2 (100 int. units); vitamin K, 0.005; nicotinic acid, 3.0; Ca pantothenate, 1.8; folic acid, 0.2; biotin, 0.02; choline bitartrate, 200.

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Reagents

For isolation of lymphocytes, we used a routine density-separation medium (Lymphocyte-M-Φ; Cedarlane Laboratories Ltd., Ontario, Canada). HNO3, 13 mol/L, and HC1O4, 9 mol/L (specially prepared reagent grade), and heparin, sodium salt (guaranteed reagent grade), were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Physiological saline was purchased from Otsuka Pharmaceuticals Co., Ltd. (Tokyo, Japan); these reagents were found to be free from manganese. MnCl2·4H2O (guaranteed reagent grade) was purchased from Wako Pure Chemical Industries, Ltd., Tokyo, Japan.

Standards were prepared by dilution of a 1000 μg/mL reference solution, a certified atomic absorption standard (Nacalai Tesque Inc.).

Procedures

Animal studies. Sprague–Dawley rats (Charles River Japan Inc., Kanagawa, Japan) were used in this study. Four-week-old female rats were assigned to each of the following two dietary groups: a purified diet containing either no manganese (manganese-free diet) or 50 μg of Mn per gram of diet (normal diet) (see Table 1). Diet and distilled water were provided ad lib. At age 12 weeks, the female rats were mated with 13-week-old males fed a commercial diet ("MF"; Oriental Yeast Co., Ltd., Tokyo, Japan). During pregnancy and lactation, the mother rats were fed the same experimental diet as before pregnancy. After weaning, the Mn-deficient and the normal male offspring were fed the manganese-free diet and normal diet, respectively, during the rest of the experimental period.

At nine weeks of age, these offspring were divided into four groups of six animals each. Group N (normal rats) and...
group M0 (manganese-deficient rats) were injected with physiological saline (NaCl 9.0 g/L) once a day for one week. Group M1 and M10 of the manganese-deficient rats were injected with manganese at 66 and 660 μg/kg body weight, respectively, in the form of MnCl₂·4H₂O in physiological saline, once a day for one week. The solutions were injected intravenously (4.0 mL/kg) over a 2-min period through the tail vein. Twenty-four hours after the last injection, the rats were anesthetized with sodium pentobarbital. Blood was collected from the aorta abdominis into a polyethylene syringe containing sodium heparin as anticoagulant (final concentration: 15 int. units/mL of blood), and the rats were killed. Then, striatum, testis, and tibia were removed, and weighed.

**Isolation of lymphocytes.** We separated lymphocytes from 5 mL of whole blood from each rat, using Lympholyte-M, as follows. Gently mix 5 mL of whole blood with an equal volume of physiological saline by inversion. Add 10 mL of lympholyte-M to a 50-mL polypropylene tube. Using a polyethylene Pasteur pipette, carefully layer 10 mL of the dilute blood over the Lympholyte-M. Centrifuge at 500 × g for 20 min at 20 °C. Very carefully discard the supernatant fluid above the lymphocyte layer, using a polyethylene Pasteur pipette. Then, carefully collect the lymphocyte layer with a polyethylene Pasteur pipette and transfer to a 10-mL polypropylene tube. Add 5 mL of physiological saline, centrifuge at 400 × g for 10 min at 4 °C to pellet the lymphocytes, and discard the supernate. Then wash three times in physiological saline before processing. Finally, suspend the lymphocyte pellet in 1.1 mL of physiological saline, then transfer exactly 1 mL of lymphocyte suspension to a 1.5-mL polypropylene tube. We then recorded the lymphocyte numbers per microliter of lymphocyte suspension, using an automated blood-cell counter. Centrifuge at 400 × g for 6 min at 4 °C and discard the supernate.

**Analysis for manganese.** Lymphocyte pellets in the 1.5-mL polypropylene tube were dissolved by adding 250 μL of 13 mol/L HNO₃ and placing each tube in a heating-block at 75 °C for 90 min. Then we measured the volume of solution, using a calibrated glass capillary pipette.

Manganese concentrations were determined with a flameless atomic absorption spectrophotometer with a graphite furnace. The instrumental conditions were as follows: wavelength, 279.5 nm; slit width, 0.38 nm; carrier gas flow rate (argon), 1.8 L/min; drying temperature, 150 °C for 20 s; ashing temperature, 650 °C for 30 s; and atomization temperature, 2500 °C for 5 s. Standards were prepared by adding manganese (0, 5, 10, 20, or 40 μg/L) to samples, which were then treated as described above. The correlation coefficients for the standard curves ranged from 0.997 to 0.999.

We calculated the manganese concentration in lymphocytes (in ng/10⁶ cells) as follows:

\[
\text{Mn in digested lymphocytes (ng/mL)} = \frac{\text{vol. of digested lymphocytes (mL)} \times 10^9}{\text{vol. of lymphocyte suspension (μL)} \times \text{no. of lymphocytes per μL of lymphocyte suspension}}
\]

One gram of whole blood was transferred into the 20-mL glass tube, then digested by adding 3 mL of 13 mol/L HNO₃ and placing each tube in a heating-block at 85 °C for 28 h. Then we measured the volume of solution. The manganese concentration was determined by flameless atomic absorb-

tion spectrophotometry as described above.

Whole tissues (striatum, testis, or tibia) were placed in the 20-mL glass tube and digested in 13 mol/L HNO₃ (striatum, 2 mL; testis, 5 mL; tibia, 5 mL) at 85 °C for 24 h, followed by addition of 9 mol/L HClO₄ (striatum, 0.4 mL; testis, 1 mL; tibia, 1 mL) and heating at 120 °C for 4 h, with a heating-block. Then we measured the volume of solution. Manganese concentrations were determined in a flame atomic absorption spectrophotometer, with the following instrumental conditions: wavelength, 279.5 nm; slit width, 0.4 nm; and gas flow rate (acetylene), 1.9 L/min. In the analysis, a linear calibration curve was obtained over the following range of concentrations: 0, 0.10, 0.25, 0.5, 1.0, and 2.0 mg/L.

Manganese concentrations in whole blood and tissues were calculated by the following formula: [Mn in digested whole blood or tissues (μg/mL) × vol. of digested whole blood or tissues (mL)]/mass of whole blood or tissues (g).

**Statistics.** The data were expressed as the mean ± SE and analyzed statistically by analysis of variance, followed by Bonferroni’s test (9) for individual differences. A value of *P* <0.05 was considered significant.

**Results.**

At the start of the study, the body weights of the rats in groups M0, M1, and M10 were lower than those in group N (Table 2). At the end of the study, the weights of these three groups had increased but remained less than that of group N (Table 2).

The manganese concentration in lymphocytes was significantly decreased in the group M0, increased in the group M10, but not significantly changed in the group M1, as compared with that in group N (Figure 1). In whole blood, the manganese concentration tended to decrease in group M0, was significantly increased in group M10, but not significantly different in group M1, as compared with group N. In striatum, testis, and tibia tissues, manganese concentrations were significantly less in group M0 than in group N. These low values were increased, in a dose-dependent manner, in groups M1 and M10.

**Discussion.**

Manganese is an essential trace element, with many biological functions (10–12), so a clear knowledge of the nutritional status of manganese is important. The simplest and most commonly used method for assessing the nutritional status of manganese during TPN is to measure manganese in plasma or serum, but these values may not always reflect the nutritional status of manganese. Injected manganese is cleared from the blood within minutes (13, 14), so manganese concentration measured in blood may

| Table 2. Changes in Body Weights of Rats during the Intravenous Injection Studies |
|---------------------------------|-----------------|-----------------|-----------------|
| Body weight, g                  | Initial         | Final           |                |
| Group N                         | 342 ± 2         | 361 ± 1         |                |
| Group M0                        | 208 ± 7         | 220 ± 9         |                |
| Group M1                        | 214 ± 12        | 232 ± 12        |                |
| Group M10                       | 213 ± 12        | 242 ± 12        |                |

All values represent mean ± SE of six rats per group. See text for definitions of groups.

*P* <0.05, **P* <0.01.
The manganese status of tissue is much better reflected by lymphocytes than by whole blood.

Therefore we suggest that manganese concentrations in lymphocytes instead of whole blood should be used for assessing the manganese nutritional status. Further studies in humans are necessary to confirm this.

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References

Fig. 1. Changes in manganese concentrations in lymphocytes and whole blood (top) and in striatum, testis, and tibia (bottom), 24 h after intravenous injection of manganese in manganese-deficient rats. Bars (left to right): group N, group M0, group M1, group M10. All values represent mean ± SE of six rats per group. #, P < 0.01.