Zinc Bioavailability Is Improved by the Micronised Dispersion of Zinc Oxide with the Addition of L-Histidine in Zinc-Deficient Rats

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Summary Zinc fortification of milk or soft drinks is usually used to combat zinc deficiencies in developing countries. Water-soluble zinc compounds, such as zinc sulfate or zinc citrate, are better absorbed but have an unacceptable taste. A micronised, dispersible zinc oxide (MDZnO), which does not have such a problem concerning taste, had higher solubility compared to ZnO (zinc oxide) in an artificial gastric solution. MDZnO was tested for its bioavailability using zinc-deficient Wistar rats. Prior to the experiment, rats were fed zinc-deficient diet for 3 wk and were orally administered control (distilled water) or zinc solutions (ZnO, ZnO+L-histidine (His), MDZnO, MDZnO+His, 1 mg zinc/kg or 3.2 mg His/kg body weight). Compared to ZnO, MDZnO showed a lag in peak time and a lengthy period of continued high plasma zinc concentration after the single oral administration of zinc compounds. Addition of His to MDZnO elevated serum zinc concentration. Serum zinc concentration (area under the curve) in rats administered MDZnO with His was significantly higher than in rats administered distilled water (p<0.05). Liver zinc level was significantly higher in rats administered MDZnO with His compared with control rats (p<0.05), although the level was not affected by the administration with ZnO alone, ZnO+His, or MDZnO alone. In conclusion, the solubility of ZnO was elevated by the micronised dispersion technique and an in vivo study using zinc-deficient rats confirmed that its bioavailability was significantly improved compared to ZnO and the coadministration of His additively enhanced the bioavailability of MDZnO.

Key Words zinc, bioavailability, fortification, trace element, histidine

Severe zinc deficiency and suboptimal zinc status have been accepted in many population groups in both less developed and industrialized countries (1). Several researchers reported that supplementation or fortification with zinc has yielded higher children’s growth (2, 3), enhanced immune function (4) and improved pregnancy outcome (5). These suboptimal zinc statuses were caused by the inhibitors of zinc absorption, phytate, in staple foods like cereals, corn and rice in many cases (6). It is important but difficult to avoid or limit these components with inhibitory effects on zinc absorption in agricultural and food processing methods.

Food fortification programmes are usually considered the most cost-effective and sustainable approach to combat zinc deficiency. Combined iron-zinc fortification regimens are employed with increasing frequency in field trials to combat co-occurring iron and zinc deficiencies. As several fortification studies raised the possibility for potential competition between iron and zinc, the bioavailability of zinc compounds should be estimated by tissue zinc level as well as serum zinc level (7).

The success of a zinc fortification programme depends largely on the careful choice of highly absorbed zinc compounds. Water soluble zinc compounds, such as zinc sulfate heptahydrate or zinc citrate, are better absorbed than zinc oxide (ZnO) (8, 9), but were reported to have an unacceptable taste in food vehicles. ZnO does not have such a problem concerning taste and is a commonly used source of supplementary zinc in diets of animals and humans in many countries such as the United States, Korea, the Philippines and EU accession states, but is not used in Japan. ZnO is water insoluble and is precipitated when used for fortifying liquid foods (10, 11).

A micronised dispersion technique has been developed for food fortification with iron. Micronised dispersible ferric pyrophosphate has less “iron taste” compared to soluble iron and the formation of agglomerates is avoided by adding emulsifiers. The high area under the curve value, a lag in peak time and continued high

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Abbreviations: ALP, alkaline phosphatase; AUC, area under the curve at 4 h after the administration; His, L-histidine; ICP-MS, inductivity-coupled plasma mass spectrometry; MDZnO, micronised dispersible zinc oxide; MPS, mean particle size; SD, standard deviation; SOD, superoxide dismutase; ZnO, zinc oxide.
serum iron concentration were observed in rats after the oral administration of micronised dispersible ferric pyrophosphate compared to ferric pyrophosphate, sodium ferrous citrate and ferrous sulfate (12). Micronised dispersible ferric pyrophosphate is well absorbed as ferrous sulfate in adults (13). We have developed a micronised dispersible zinc oxide (MDZnO, SunActive Zn®; Taiyo Kagaku (Yokkaichi, Japan), which completely disperses, without precipitation, insoluble zinc in liquid formulations.

In this study, we hypothesized that, similar to the observation of micronised dispersible ferric pyrophosphate in men (13) and rats (12), oral administrations of MDZnO would result in highly increased bioavailability and tissue concentration of zinc compared to the administration of ZnO. We estimated the solubility of MDZnO in an in vitro study using an artificial gastric solution, since the bioavailability of a trace element highly depends on its solubility (14, 15). Furthermore, we performed two in vivo experiments concerning the time-dependent changes of serum zinc concentration after a single administration of zinc compounds (Experiment 1) and restoration of tissue zinc concentrations after 7 d administration of zinc compounds (Experiment 2). In both in vivo experiments, the interaction of coadministered L-histidine (His) with bioavailability of either ZnO compound (micronised or not micronised) was investigated, because His, a zinc chelator, is used in an effort to enhance zinc bioavailability.

**MATERIALS AND METHODS**

**Zinc compounds.** The zinc compounds tested are shown in Table 1. The ZnO compound was purchased from Honjo Chemical Corporation (Osaka, Japan). His was purchased from Kanto Chemical Co. Ltd. (Tokyo, Japan). Artificial gastric juice (0.2% NaCl, 2.4% HCl, pH 1.2) used to measure the solubility of zinc compounds is described elsewhere (16).

**Solubility of zinc compounds.** Zinc compounds were solved in artificial gastric juice at the zinc concentration of 100 mg/L (final volume: 50 mL). The solutions were incubated at 37°C with modest shaking and a portion (3 mL) of the solution was repeatedly sampled at 0, 30, 60 and 120 min after the preparation of the zinc solution. Each fraction was passed through a 0.1 μm membrane filter (Millipore) and was autoclaved for 15 min at 121°C to obtain a uniform solution containing soluble zinc compounds. Zinc concentrations were measured by inductively-coupled plasma mass spectrometry (ICP-MS, Elan DRC II, PerkinElmer, Inc., MA).

**Animals and diets.** All procedures were performed in accordance with the Animal Experimentation Guidelines of Nagoya University. The bioavailability of the zinc compounds was determined by the serum and tissue zinc concentration after oral administration of zinc compounds. Forty-seven male Wistar rats (CLEA Japan, Inc., Tokyo, Japan) weighing 200 g were housed individually in stainless cages and kept under controlled conditions with a daily 12-h light:dark cycle. Millipore water (Simpli Lab-UV, Millipore) was provided for all rats ad libitum throughout the whole study. All of the rat diets used in the study were prepared by CLEA. The zinc-deficient diet was based on a modified AIN-93 formula (17) in which an AIN-93 mineral mixture with zinc omitted was used. Zinc concentration of the zinc-deficient diet was measured to be 2.66 mg Zn/kg diet by ICP-MS. Rats consumed a zinc-deficient diet ad libitum for 21 d. During this depletion period, the rats were weighed and four randomly chosen rats had their blood collected intraorbitally under an ether anesthesia into heparin-coated tubes for serum zinc and alkaline phosphatase (ALP) determination. Rats were randomly assigned to 2 groups of 10 and 37 rats and provided for the following two experiments.

**Experiment 1. Serum zinc level after a single oral zinc administration.** This method was based on plotting the serum iron concentrations after oral administration of iron compounds to rats (12, 18). Prior to the experiment, ten rats were fed with a zinc-deficient diet for 3 wk, as described in the previous section. They were anesthetized under pentobarbital and cannulated in the jugular vein and were orally administered one of the four solutions containing ZnO compounds (ZnO, ZnO+His, MDZnO, MDZnO+His, 1 mg zinc/kg or 3.2 mg His/kg body weight) or Millipore water (Control) on and after the day following surgical operation. After the oral administration of the zinc solution, blood was collected from the cannulation of the jugular vein before and at 30, 60, 120, and 240 min after the administration. Administrations of zinc solution were repeated up to 5 times at 3–4 d intervals over 3 wk (ZnO [n=4], ZnO+His [n=3], MDZnO [n=4], MDZnO+His [n=4], control [n=4]). Collected sera were kept under −80°C until the measurement of zinc concentration. The serum zinc concentrations were measured by the ICP-MS described in the following section. The area under the curve at 4 h after the administration (AUC4) values for various zinc compounds were calculated.
using the trapezium rule. For all zinc compounds, we plotted the increment above 0 mg/L as the basis for calculating AUC₄. The dimensions of the AUC₄ are (mg·h)/L (19).

Experiment 2. Tissue and serum zinc level after 7 d of repeated zinc administration. Prior to the start of the experiment, 37 rats were fed a zinc-deficient diet for 3 wk as described in the above section. They subsequently consumed the same zinc-deficient diet, but were orally administered a zinc solution containing one of the four ZnO compounds (ZnO [n=8], ZnO+His [n=8], MDZnO [n=8], MDZnO+His [n=8]) or Millipore water (n=5) used in Experiment 1 for 7 d. Individual food consumption was measured twice a week throughout the experimental period. After the administration period, rats were weighed, blood was collected from the inferior vena cava for serum zinc, ALP, superoxide dismutase (SOD) determination, and the rats were killed under ether and pentobarbital anesthesia. Serum and tissues were kept under −80°C until the measurement of zinc analysis.

Zinc analysis. About 100 mg of tissues and serum were mineralized after the addition of 2.5 mL concentrated nitric acid for the analysis of poisonous metal (Wako Pure Chemical Industries, Ltd., Osaka, Japan) by using a microwave digestion system with temperature control (Multiwave, PerkinElmer Life Sciences) in a polytetrafluoroethylene tube. Digested samples were quantitatively diluted, and ⁶⁵Zn was measured by ICP-MS. The reference material was bovine liver (SRM 1577b, NIST) and durum wheat flour (RM 8436, NIST), which was analyzed with the samples for quality control of the zinc analysis. Each sample was analyzed in duplicate for zinc concentration.

Statistical analysis. Data are presented as means±SD. One-way ANOVA with Tukey's post hoc test were used to compare the values among 5 groups, including the control. Two-way ANOVA was used to compare zinc concentrations in serum (AUC₄) and tissues among 4 groups, excluding control in Experiments 1 and 2; namely, the effect of micronised dispersion on the bioavailability of zinc in MDZnO, the effect of His addition on the bioavailability of zinc in MDZnO and the interaction between the micronised dispersion and His addition were analysed. Differences were regarded as significant at p<0.05. All the statistical analyses were performed by the statistical software Prism ver 4.03 (GraphPad Software, Inc., San Diego, CA).

RESULTS

Solubility of zinc compounds

The solubility of MDZnO was higher than 80% before the incubation in the artificial gastric juice and reached 95% after 2 h of incubation at 37°C. On the other hand, the solubility of ZnO was about 70% before the incubation and reached 95% after 2 h of incubation at 37°C (Fig. 1).

Plasma zinc level and ALP activity during zinc deficient period

The serum zinc level of rats fed the zinc-deficient diet was significantly decreased during the first 2 wk of feeding the zinc-deficient diet and was lower than 0.7 mg/L, which was the index of Zn deficiency. Serum ALP activity declined abruptly during the first week of feeding the zinc-deficient diet and rose gradually during the subsequent zinc-deficient period, but the serum ALP activity after 2 d of zinc-deficient period was significantly lower than the value before the zinc-deficient period (Fig. 2).

Experiment 1. Serum zinc level after a single oral zinc administration

Serum zinc levels of rats were increased after a single oral administration of all solutions containing zinc compounds. The elevations of plasma zinc levels caused by MDZnO or MDZnO+His solution were sustained for about 4 h, while those elevations caused by ZnO reverted to the initial level 2 h after zinc administration (Fig. 3A). Serum zinc levels for MDZnO+His and MDZnO rats were significantly higher than for the control at 2 h after the administration (p<0.01 and p<0.05, respectively).

AUC₄ of serum zinc level was increased by 22.38. 46 and 66% of control values following a single oral administration of ZnO, ZnO+His, MDZnO and MDZnO+His, respectively, providing evidence of the effectiveness of the micronised dispersion technique and His addition (Fig. 3B). The AUC₄ of MDZnO+His
body weight or food intake. There were no significant differences in body weight gain, final body weight, food intake or zinc intake during 4 wk of the experimental period (Table 2). The weight of the pancreas of rats administered zinc solutions excluding ZnO were significantly higher than rats administered the control solution. There were no significant differences in the other tissue weights among the groups (Table 3).

The relative biological value was calculated relative to ZnO from the AUC4 of the serum zinc level. The relative biological value was 1.0, 1.13, 1.19 and 1.36 of ZnO values following a single oral administration of ZnO, ZnO+His, MDZnO and MDZnO+His, respectively. Experiment 2. Tissue and serum zinc level and enzyme activities after repeated zinc administration

Administration of all four zinc solutions did not affect body weight or food intake. There were no significant differences in body weight gain, final body weight, food intake or zinc intake during 4 wk of the experimental period (Table 2). The weight of the pancreas of rats administered zinc solutions excluding ZnO were significantly higher than rats administered the control solution. There were no significant differences in the other tissue weights among the groups (Table 3).

The liver zinc level was significantly higher in rats administered MDZnO with His compared with control rats (*p<0.05), although the level was not affected by the administration of ZnO alone, ZnO+His, or MDZnO alone. By two-way ANOVA, the effects by His addition and the interaction effect between the micronised dispersion technique and His addition were significant (p<0.02 and p<0.05, respectively), while the effect

Table 2. Body weight gain, final body weight, food intake and zinc intake.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ZnO</th>
<th>ZnO+His</th>
<th>MDZnO</th>
<th>MDZnO+His</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain (g/28 d)</td>
<td>123.46±5.14</td>
<td>129.30±16.46</td>
<td>126.10±13.49</td>
<td>125.00±13.92</td>
<td>129.30±6.80</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>318.96±7.12</td>
<td>324.00±17.74</td>
<td>321.00±14.56</td>
<td>321.34±18.15</td>
<td>327.43±8.24</td>
</tr>
<tr>
<td>Food intake (g/d)</td>
<td>17.37±0.67</td>
<td>17.56±1.37</td>
<td>17.63±1.24</td>
<td>17.35±0.78</td>
<td>17.76±0.69</td>
</tr>
<tr>
<td>Zinc intake (µg/d)</td>
<td>46.20±1.79</td>
<td>46.70±3.65</td>
<td>46.90±3.30</td>
<td>46.14±2.07</td>
<td>47.24±1.82</td>
</tr>
</tbody>
</table>

Values are means±SD, n=8 for ZnO, ZnO+His, MDZnO and MDZnO+His, n=5 for control.

Table 3. Tissue weight of rats that were orally administered zinc solution for a week in Experiment 2 (g/100 g body weight).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ZnO</th>
<th>ZnO+His</th>
<th>MDZnO</th>
<th>MDZnO+His</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>4.20±0.11</td>
<td>4.26±0.20</td>
<td>4.24±0.24</td>
<td>4.09±0.54</td>
<td>3.91±0.12</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.59±0.03</td>
<td>0.62±0.04</td>
<td>0.63±0.03</td>
<td>0.64±0.05</td>
<td>0.62±0.02</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.13±0.07</td>
<td>0.18±0.04</td>
<td>0.20±0.02</td>
<td>0.22±0.04</td>
<td>0.21±0.00</td>
</tr>
<tr>
<td>Testis</td>
<td>0.94±0.05</td>
<td>0.93±0.05</td>
<td>0.98±0.05</td>
<td>0.97±0.08</td>
<td>0.88±0.11</td>
</tr>
<tr>
<td>M. Quadriceps</td>
<td>1.27±0.05</td>
<td>1.22±0.05</td>
<td>1.28±0.03</td>
<td>1.32±0.06</td>
<td>1.29±0.11</td>
</tr>
<tr>
<td>M. Gastrocnemius</td>
<td>1.13±0.06</td>
<td>1.14±0.04</td>
<td>1.16±0.05</td>
<td>1.14±0.08</td>
<td>1.19±0.06</td>
</tr>
<tr>
<td>Femur</td>
<td>0.66±0.02</td>
<td>0.64±0.02</td>
<td>0.66±0.03</td>
<td>0.63±0.03</td>
<td>0.63±0.02</td>
</tr>
</tbody>
</table>

Values are means±SD, n=8 for ZnO, ZnO+His, MDZnO and MDZnO+His, n=5 for control. Values with different superscripts are significantly different (p<0.05).
from the micronised dispersion technique was not significant in the liver zinc level. Similarly, kidney and pancreas zinc levels were also significantly higher in rats administered MDZnO with His compared with the control \((p<0.05)\), and the increase of zinc level in rats administered ZnO was smaller than that in rats administered MDZnO with His. By two-way ANOVA, the effect for His addition was significant \((p<0.01)\), while the effect for the micronised dispersion technique and the interaction effect between the micronised dispersion technique and His addition were not significant in the kidney zinc level.

In contrast, femur and serum zinc levels were higher in rats administered either ZnO, ZnO+His, MDZnO, or MDZnO+His, and there were no differences in zinc levels among these four groups. No significant elevation in zinc level was observed in testis, gastrocnemius muscle, or quadriceps muscle after the zinc administration (Table 4).

Slight but insignificant increases in serum ALP and SOD activities were observed after the administration of all the zinc compounds compared to the control. However, there were no differences in these enzyme activities among the four solutions containing zinc compounds (Table 5).

**DISCUSSION**

Water solubility is an important factor influencing mineral availability (15). Particle size can be an important determinant of the solubility of trace elements (20). Decreasing the particle size of elemental iron powders by 50–60%, to a mean particle size (MPS) of 7–10 μm, increases iron absorption by ~50% in rats (21, 22). In a human study, iron absorption from hydrogen-reduced elemental iron with particle sizes between 5 and 10 μm was comparable to that from iron sulfate (23). In a human stable isotope study, the relative biological value of a dispersible ferric pyrophosphate with a MPS of 0.5 μm was comparable to that of ferrous sulfate (13). Particle size has previously been shown to have a positive influence on iron absorption. Motzok et al. (21) found about a three-fold increase in bioavailability of reduced iron by reducing the particular size from 24–40 μm to 7–10 μm. Similar observations were also reported for electrolytic iron powder (24), ferric orthophosphate (24) and micronised dispersible ferric pyrophosphate (12). Concerning zinc compounds, decreasing MPS would increase zinc solubility, as observed in iron (20) and calcium (25).

Solubility of zinc is increased when zinc forms a complex between a low-molecular-weight ligand or chelator and that complex can be absorbed. His is a good chelator of zinc, and clinical studies in human subjects have shown a positive effect of His on zinc absorption as measured by the increase in serum zinc area under the curve (26, 27). In the present study, the coadministration of His \((Zn : His = 1:3.2)\) elevated the serum zinc concentration after oral administration of MDZnO. The ratio of His to zinc is important because it may have a strong effect on zinc metabolism. High doses of His were used earlier to induce experimental zinc deficiency in human subjects, because they enhance the urinary excretion of zinc (28). Ingestion of a zinc complex with His at a ratio of 1:12 increased serum zinc concentration and urinary zinc excretion. Ingestion at a ratio of 1:2 showed less increase in urinary zinc excretion.
although it increased serum zinc concentration.

Stable isotopes were not used in the present study to estimate the bioavailability of zinc compounds (Experiments 1 and 2). We used zinc-deficient rats to decrease the internal zinc storage and to detect the effects of orally administered zinc compounds. When using stable or radioisotope techniques it is quite important that the physical and chemical properties of the labeled compounds are comparable with those of their commercial counterpart. In the present study, it was difficult to prepare a labeled compound with physical and chemical properties similar to commercially available MDZnO. This was mainly caused by the larger particle sizes of commercially available labeled ZnO compounds compared to those of unlabeled ZnO used for producing MDZnO. The labeled MDZnO, which was made as a research prototype using a down-scaled manufacturing procedure similar to the commercial production procedure were not used in the present study because of its larger particle-size distribution compared to the commercial MDZnO.

The serum zinc concentration after oral administration of MDZnO showed a lag in peak time and a lengthy period of continued high concentration, which suggest sustained release of zinc in the serum. Furthermore, the high AUC₄ value for MDZnO could contribute to its high absorption and bioavailability. The lag in peak time and the sustained release of zinc from MDZnO is probably a consequence of encapsulation with emulsifiers. In a previous study concerning iron ([12]), the lag in peak time and the sustained release of iron were observed after the oral administration of micronised dispersible ferric pyrophosphate to rats. The peak times of serum iron level were between 30 and 60 min after the oral administration of ferric pyrophosphate, sodium ferrous citrate, and ferrous sulfate. But the peak time of serum iron levels were 2 h after the oral administration of micronised dispersible ferric pyrophosphate. In the previous study concerning micronised dispersible docosa-hexaenoic acid, the lag in peak time and the sustained release were observed in male subjects (Ishikawa M et al., Proceeding of Japanese Society of Nutrition and Food Science, 2B-15a, 2002).

Absorbed zinc was transported to the liver via a portal system and about 70% of absorbed zinc was stored in the liver 5 to 48 h after administration. Liver zinc levels gradually decrease while, on the other hand, zinc levels increase in the central nervous system, bone and skeletal muscle. Liver zinc levels were not increased after the administration of any zinc solution in the present study. Liver zinc content was hardly affected by the dietary zinc concentration ([29]). These previous findings are consistent with the present observation that the elevation of liver zinc levels was not observed in any of the groups except for the significant elevation after the administration of MDZnO with His. As shown in Fig. 3, the administration of MDZnO with His significantly elevated the serum zinc level (AUC₄). Further studies are required to clarify the mechanism for the significant elevation of the liver zinc level after the administration of MDZnO with His.

The bone zinc level is often used as an index for the estimation of orally administered zinc bioavailability after administration for 3 to 5 wk ([29–33]). In the present study, all the zinc solutions significantly increased femur zinc levels compared to water, but there were no differences among zinc solutions. The reason is not obvious but zinc solutions were administered for only 1 wk, which is quite shorter than in the previous studies. Therefore a longer administration period would be required to estimate the effect of MDZnO with His on femur zinc levels.

Dietary inhibitors of zinc absorption such as phytate and dietary fiber are likely the most common causative factors concerning suboptimal zinc status ([6]). It is therefore important to estimate the zinc fortification under the existence of gastrointestinal content. In the present study, zinc solutions were administered to rats freely accessing a zinc-deficient diet. Therefore elevations of serum zinc after the administration of MDZnO with His could be observed under the existence of gastrointestinal content. Further research is required to estimate the effect of MDZnO with His as a component of the solid diet, especially one high in phytate.

Acknowledgments

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