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Influence of Long-term Enteral Nutrition on Pharmacokinetics of Digoxin in Rats

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Summary: This study was designed to clarify the influence of long-term enteral nutrition (EN) on the pharmacokinetics of digoxin. Rats were fed EN diets (semi-digested, digested, and elemental) for 4 weeks, then digoxin (0.05 mg/kg) was administered orally. The AUC₀–∞ and kₚ of digoxin were significantly reduced in the semi-digested diet group versus the control, while the AUC₀–∞ was significantly increased in the digested and elemental diet groups. The mRNA level of Slco1a4 was significantly reduced at the upper small intestine in all EN groups. Further, the expression levels of P-glycoprotein (P-gp) protein and Abcb1a mRNA were increased at the same site in all EN groups, and the increases were significant in the elemental diet group. Cyp3a2 protein and mRNA expressions were significantly reduced in the liver in the digested and elemental diet groups. Abcb1a mRNA was also significantly reduced in the kidney in these groups. These results indicate that the absorption kinetics at the small intestine is influenced by semi-digested diet, and the elimination kinetics in the liver and kidney are influenced by digested and elemental diet. Semi-digested diet also altered digoxin pharmacokinetics in humans. Thus, the effect of long-term EN on digoxin pharmacokinetics depended on the dietary components.

Keywords: absorption; Cyp3a2; digoxin; enteral nutrition; metabolism; Oatp; P-gp; pharmacokinetics

Introduction

Enteral nutrition (EN) is used for patients whose nutritional requirements cannot be met by ingestion, but who retain adequate gastrointestinal function. For example, it is frequently used in patients with inflammatory bowel disease (IBD), such as Crohn’s disease, or esophageal obstruction. EN is recognized worldwide as inexpensive and safe,¹,² and guidelines for its use are well established.³

Diet formulations used for EN are categorized into semi-digested diet, digested diet, and elemental diet. Termeal⁵ is a liquid semi-digested diet, and its nutritional composition is similar to that of normal diet, with milk protein and casein as nitrogen sources. Termeal⁵ includes some fiber (0.3 g/100 kcal). On the other hand, digested and elemental diets differ in composition from normal diet. Peptino⁶ is a liquid digested diet, which contains di- or tripeptides as a nitrogen source, and does not include fat. Elental⁷ is a liquid elemental diet, which includes amino acids as a nitrogen source, and contains some fat. Peptino⁶ and Elental⁷ do not include fiber, and Termeal⁵ includes little fiber.

The effects of EN diets on the gastrointestinal tract have been studied in rats, and changes of small-intestinal mucosal weight and total protein have been observed.⁴,⁵ It is also well known that enteric bacteria and endotoxin are transferred from the gastrointestinal tract to blood and organs, so-called bacterial translocation, when the gastrointestinal tract is not used for a long period, e.g. during total parenteral nutrition (TPN).⁶-⁸ Bacterial translocation also occurs during EN due to atrophy of the small-intestinal mucosa.⁹-¹¹

There have been a few reports on the influence of EN on drug pharmacokinetics, but these were all studies of drug-food interaction, involving single administration of an EN diet containing the investigational drug.¹²-¹⁴ The effect of different EN diet compositions on drug pharmacokinetics has not been examined.

In this study, we focused on the effect of long-term EN on the pharmacokinetics of digoxin as a model drug. Digoxin is a cardiac...
glycoside, which produces a cardiotoxic effect by inhibition of Na\(^+\)/K\(^+\) ATPase in cardiomyocytes. It is clinically used to treat cardiac failure, especially combined with atrial fibrillation, and is often administered for patients who require EN. However, Rathore et al. reported that the therapeutic range of serum digoxin concentration was only 0.5–0.8 ng/mL for male patients whose left ventricular ejection fraction was less than 45%, and noted that the mortality rate was increased when the serum digoxin concentration was above this range.\(^\text{15}\) Further, the inotropic effect of digoxin is influenced by extracellular potassium concentration; if the extracellular potassium concentration is reduced, the inotropic effect of digoxin may be increased and digoxin toxicity may occur, even though the serum digoxin concentration remains in the therapeutic range.\(^\text{16-18}\)

We hypothesized that EN diets with different compositions would differently influence digoxin’s pharmacokinetics, and so might alter the serum level of digoxin and potentially influence the outcome of digoxin treatment. To test this idea, we examined the effect of long-term EN with three kinds of diets (semi-digested, digested and elemental) on the pharmacokinetics of digoxin and the expression of digoxin transporter and metabolic enzyme genes in male Sprague-Dawley rats. We also researched retrospectively the effect of semi-digested diet on the pharmacokinetics of digoxin in humans.

**Methods**

**Materials:** Male Sprague-Dawley rats (200–220 g body weight) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Calorie Mate\(^\text{®}\) was purchased from Otsuka Pharmaceutical, Inc. (Tokyo, Japan) and was used as the control diet. Termeal\(^\text{®}\) and Peptino\(^\text{®}\) were purchased from Terumo, Inc. (Tokyo, Japan) and were used as the semi-digested and digested diets, respectively. Elental\(^\text{®}\) was purchased from Ajinomoto, Inc. (Tokyo, Japan) and was used as the elemental diet. Diamine oxidase from porcine kidney, cadaverine dihydrochloride, peroxidase from horseradish, and sodium diethyldithiocarbamate were purchased from Sigma-Aldrich Japan, Inc. (Tokyo, Japan). DA-67 and ascorbate oxidase were purchased from Wako Pure Chemical Industries, Inc. (Osaka, Japan). Digoxin powder (0.1%) was purchased from Chugui Pharmaceutical, Inc. (Tokyo, Japan). Heparin sodium was purchased from Mochida Pharmaceutical, Inc. (Tokyo, Japan). Pentobarbital and diethyl ether were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals were of analytical or high-performance liquid chromatography (HPLC) grade. Primers used for quantitative real-time polymerase chain reaction (PCR) were synthesized by Invitrogen Life Technologies Japan, Inc. (Tokyo, Japan). All animal procedures were carried out in accordance with the standards set forth in the Guidelines for the Care and Use of Laboratory Animals at the Takara-machi campus of Kanazawa University.

**Construction of long-term enteral nutrition model rats:** Male Sprague-Dawley rats, 6 weeks old, were divided randomly into four groups (n = 4 each) and allowed free access to EN diet and water. Control rats were fed Calorie Mate\(^\text{®}\). The semi-digested diet group rats were fed Termeal\(^\text{®}\), while the digested diet group rats received Peptino\(^\text{®}\) and the elemental diet group rats received Elental\(^\text{®}\). All of these diets, including the control, are manufactured for human use. Rats of the EN groups received EN from water bottles. EN diets were replaced with fresh diets every day. Rats were used for experiments after 4 weeks.

**Measurement of body weight and tissue preparation:** Rat body weight was measured weekly from 6 to 10 weeks of age (at the start of, and during, EN feeding). Blood was collected from the femoral vein under pentobarbital (30 mg/kg) anesthesia at 2 and 4 weeks after the start of EN feeding. After EN feeding, the animals were killed humanely and liver, kidney, fat, small intestine, spleen, heart, and testis were collected and weighed. The first 5 cm of the proximal intestine, the last 5 cm of the distal intestine, and the rest of the small intestine were used as the upper, lower, and middle parts of the small intestine, respectively. Portions of liver, kidney and small intestine (central regions of the upper, middle, and lower small intestine) were stored at −80°C until required for RNA extraction. Cryosections were prepared from liver and small intestine using a microtome and stained with hematoxylin-eosin (HE) and Oil red O.

**Measurement of plasma diamine oxidase activity:** Plasma diamine oxidase (DAO) activity was measured using the method reported by Takagi et al.\(^\text{19}\) Substrate solution (cadaverine solution, 30 mM, 1.5 mL) was incubated in a test tube at 37°C for 5 min, then 0.1 mL of sample serum was added and mixed. Incubation was carried out at 37°C for 30 min. Then, 1.5 mL of color solution [100 μM DA-67, 6 units/mL peroxidase (POD), and 5 units/mL ascorbate oxidase (ASOD)] was added to the test tube and mixed, and incubation was continued at 37°C for 60 min. Finally, 0.05 mL of stop solution (sodium diethyldithiocarbamate, 30 mM) was added and mixed, and the resulting mixture was stored at room temperature until DAO measurement. Serum DAO activity was measured in terms of absorbance at 668 nm using a BIOSPEC-1600 (Shimadzu Co., Kyoto, Japan).

**Measurement of plasma digoxin concentration:** After the 4-week EN treatment period, the rats were anesthetized with pentobarbital, and digoxin (0.05 mg/kg) dissolved in distilled water was orally administered via a gastric tube. Blood samples (300 μL each) were collected from the femoral artery via a heparin-coated cannula (polyethylene tubing SP31) at 0, 10, 30, 40, 60, 90, 120, 180, 240 and 300 min (total 10 time points). The blood samples were taken into plastic tubes containing 30 μL (100 units/mL) heparin sodium, and centrifuged at 14,000 rpm, 4°C for 5 min. Plasma was collected and stored at −80°C until analysis of digoxin concentration.

Plasma digoxin was measured quantitatively by using the digoxin kit of the Dimension\(^\text{®}\) clinical chemistry system (Siemens Japan K.K., Tokyo, Japan) with Dimension\(^\text{®}\) Xpand plus-HM (Siemens Japan K.K.). This method was validated between 0.06 ng/mL and 5.00 ng/mL with the mean overall values of calculated accuracy and precision (percent) of 15% and 10%, respectively. The samples were measured after dilution when the plasma digoxin concentration was above 5.00 ng/mL.

**Analysis of digoxin pharmacokinetics:** One-compartment analysis including a first-order absorption process was employed to estimate the pharmacokinetics parameters (T\(_{\text{max}}\), C\(_{\text{max}}\), V\(_{\text{d}}\), k\(_{\text{p}}\), k\(_{\text{e}}\), AU(C\(_{\text{max}}\), and CL\(_{\text{uc}}\)) of digoxin, using WinNonlin software version 6.0.1 (Bellkey Sciences G.K., Tokyo, Japan).

**Quantitative real-time PCR analysis:** Total RNA was isolated from each part of the small intestine (upper, middle and lower), kidney and liver using a Gen Elute\textsuperscript{TM} Mammalian Total RNA Miniprep Kit (Sigma-Aldrich Japan Co.) according to the manufacturer’s instructions. The cDNA was prepared by reverse transcription of each RNA sample at 37°C for 2 h with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Copyright © 2013 by the Japanese Society for the Study of Xenobiotics (JSSX).
Foster City, CA, USA). Quantitative real-time PCR was performed on an Mx3000P® Real-Time QPCR System (Agilent Technologies, Inc., Santa Clara, CA, USA). The following primers were used for amplification of rat Slco1a4 (Oatp1a4): forward 5'-TCT CAT CAG GGG GCT TCA ATG GCT TAG-3' and reverse 5'-AAT TGA TTT GVA ATG GGG CAT GCA CAA TTA A-3' (259 bp), Slco1a5 (Oatp1a5): forward 5'-ATG CTT CTA CTT GGG GTA TTT TAG GAT TAT-3' and reverse 5'-CCA TGG CTG AGG ACA CAT G-3' (151 bp), Abcb1a (Mdr1a): forward 5'-TGA ACT GTG ACC ATG CGA GAT GTT AAA TA-3' and reverse 5'-GTC TCT TCT GAA GAC TCT AAA ATG AGC ATG-3' (153 bp), Abcb1b (Mdr1b): forward 5'-CCA GGA GAG AAG ACT TAG TTT GTC-3' and reverse 5'-GGC AAA CAC TGG TTG TAT GCA C-3' (157 bp), Cyp3a2: forward 5'-AGT AGT GAC GAT TCC AAC ATA T-3' and reverse 5'-TCA GAG GTA TCT GTG TGT CCT-3' (252 bp), Actb (β-actin): forward 5'-TGA GCG CAA GTC CTT TGT GTG CAT-3' and reverse 5'-TAG AAG CAT TGG CGG TGC ACG ATG-3' (129 bp), respectively. The PCR conditions were one cycle at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

Each sample of cDNA was diluted with Easy Dilution Solution (Takara Bio Inc., Shiga, Japan) to three different concentrations. The standard curve for the real-time PCR analysis was generated as described previously.40 In brief, a gel-purified PCR product that had been quantified by measuring the UV absorbance (A260) was diluted to 1 × 10^3 to 1 × 10^6 copy/µL and used as the standard. The copy number was calculated based on the conversion formulas: 1 A260 unit of ssDNA = 33 µg/mL H2O, average molecular weight of cDNA = number of bases × 330 Da. The efficiency of the reverse-transcription was assumed to be 100%. mRNA expression is given as the copy number per unit RNA amount (copy/µg total RNA). Transcription was assumed to be 100%.

**Western blot analysis:** The intestinal crude membrane fraction was prepared according to the method of McCullagh et al. with slight modifications.21 In brief, a small portion of mucosa of the small intestine was suspended in a buffer consisting of 210 mM sucrose, 2 mM EGTA, 40 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 30 mM HEPES, pH 7.4, and homogenized by using a Polytron-type homogenizer. Then, 2 mL of homogenate was mixed with 1.5 mL of 1.17 M KCl solution containing 58.3 mM tetrasodium pyrophosphate, kept on ice for 15 min, and centrifuged at 230,000 × g for 75 min. The resultant pellet was suspended in a buffer consisting of 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA and centrifuged again at 230,000 × g. The obtained pellet was resuspended in the same buffer and ultrasonically dispersed. Then 4% SDS was added. A clear supernatant was obtained after centrifugation at 15,000 × g.

Hepatic microsomes were prepared by differential centrifugation. In brief, the liver was homogenized in 0.1 M KCl, 0.1 M Tris-HCl and 1 mM EDTA (pH 7.4), and the homogenate was centrifuged at 9,000 × g for 15 min. The supernatant was subjected to two additional centrifugation steps at 100,500 × g for 60 min and suspended in TGE buffer containing 20% glycerol, 10 mM Tris-HCl and 1 mM EDTA (pH 7.4). The microsomal fraction was stored at −80°C until needed.

Protein concentration was determined by means of BCA (Thermo Fisher Scientific, Waltham, MA, USA) assay according to the manufacturer’s protocol. Proteins (20 µg/lane) were separated by SDS-PAGE (7.5% polyacrylamide gel), and transferred onto a polyvinylidine difluoride membrane (Millipore Corporation, Billerica, MA, USA) at 80 V for 180 min. Ponceau S staining confirmed equal efficiency of transfer to the membrane (data not shown). For detection of rat P-gp, Cyp3a2 and β-actin proteins, the membrane was incubated in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T) and 5% skim milk for blocking, and then incubated overnight at 4°C with primary antibody in PBS-T. The membranes were then washed five times with PBS-T, and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Bands were visualized by using the enhanced chemiluminescence detection method with 20× LumiGLO® Reagent and 20× Peroxide (Cell Signaling Technology, MA, USA). Experiments were repeated at least two times and 3-4 independent lanes of blot were quantitated by densitometry using a LAS-4000 (Fujifilm, Tokyo, Japan). The primary antibodies used were mouse C219 monoclonal antibody (Covance, NJ, USA), rabbit anti-CYP3A2 antibody (Enzo Life Sciences, NY, USA) and anti-β-actin antibody (Cell Signaling Technology). The secondary antibodies were horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Cell Signaling Technology).

**Retrospective research on the pharmacokinetics of digoxin in humans:** In this retrospective database study, we examined the records of patients treated at the Kanazawa University Hospital (Kanazawa, Japan) during the period 2006–2010. We identified 677 patients who had received digoxin therapy and whose plasma digoxin concentration had been measured at our institution. We excluded patients who had received hemodialysis and those who were less than 5 years old. Finally, 568 patients were included in this retrospective study. The following parameters were recorded: age, sex, body weight, serum creatinine (sCr), diet form (normal diet or semi-digested diet), dose of digoxin, and steady-state concentration of digoxin. The creatinine clearance (CrCl) was calculated by means of the Cockcroft-Gault equation to take account of differences in skeletal muscle level. To examine the influence of diet form, we compared the concentration/dose (C/D) ratio of digoxin in the normal diet group and semi-digested diet group.

This study was approved by the ethics committee of Kanazawa University Hospital (No. 996).

**Statistical analysis:** Statistical analysis was performed with GraphPad Prism 5J (GraphPad Software Inc., San Diego, CA, USA). The unpaired Student’s t-test was performed for comparison between two independent groups. For multiple comparisons, one-way ANOVA (analysis of variance) with Dunnett’s post hoc test was performed for each group. The criterion of significance was taken to be p < 0.05.

**Results**

**Body and tissue weight:** Rats were fed Calorie Mate® (control), Termenal®, Peptinio® or Elental® for 4 weeks. Although there was no significant difference of mean body weight at 1 to 3 weeks (data not shown), the mean body weight of the semi-digested diet group (396 ± 12 g, mean ± S.E., n = 4) was significantly increased versus the control group (344 ± 24 g, mean ± S.E., n = 4) at 4 weeks. Because good growth was observed in all groups of rats, we considered these rats to be appropriate as long-term EN models.

Tissue weights (g/kg body weight) of all groups of rats at 4 weeks are shown in Table 1. At 4 weeks, the liver weight was significantly increased in the digested diet group versus the control group (p < 0.05). The liver weight was also increased at 2 weeks in this group (data not shown). Small intestine weight was signifi-
The tissue weights of rats given various kinds of enteral nutrition for 4 weeks

<table>
<thead>
<tr>
<th></th>
<th>Control diet</th>
<th>Semi-digested EN diet</th>
<th>Digested EN diet</th>
<th>Elemental EN diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>36.7 ± 5.4</td>
<td>37.8 ± 3.0</td>
<td>49.1 ± 7.7*</td>
<td>35.1 ± 5.0</td>
</tr>
<tr>
<td>Kidney</td>
<td>6.49 ± 0.99</td>
<td>8.19 ± 0.07</td>
<td>6.35 ± 0.41</td>
<td>6.41 ± 0.99</td>
</tr>
<tr>
<td>Visceral fat</td>
<td>24.4 ± 3.7</td>
<td>22.5 ± 7.9</td>
<td>21.5 ± 2.8</td>
<td>25.9 ± 1.6</td>
</tr>
<tr>
<td>Small intestine</td>
<td>21.2 ± 1.7</td>
<td>20.0 ± 1.7</td>
<td>22.2 ± 1.0</td>
<td>17.9 ± 1.3*</td>
</tr>
<tr>
<td>Upper</td>
<td>2.04 ± 0.36</td>
<td>1.83 ± 0.13</td>
<td>1.65 ± 0.08</td>
<td>1.56 ± 0.21*</td>
</tr>
<tr>
<td>Middle</td>
<td>17.7 ± 1.4</td>
<td>16.7 ± 1.3</td>
<td>19.5 ± 1.0</td>
<td>15.7 ± 1.1</td>
</tr>
<tr>
<td>Lower</td>
<td>1.48 ± 0.08</td>
<td>1.45 ± 0.26</td>
<td>0.99 ± 0.12*</td>
<td>0.65 ± 0.17*</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.48 ± 0.34</td>
<td>1.41 ± 0.12</td>
<td>1.80 ± 0.07</td>
<td>1.98 ± 0.18*</td>
</tr>
<tr>
<td>Heart</td>
<td>3.32 ± 0.28</td>
<td>3.28 ± 0.41</td>
<td>3.38 ± 0.11</td>
<td>3.19 ± 0.15</td>
</tr>
<tr>
<td>Testis</td>
<td>10.3 ± 0.9</td>
<td>8.56 ± 1.00*</td>
<td>9.32 ± 0.51</td>
<td>8.47 ± 0.27</td>
</tr>
</tbody>
</table>

Tissue weights are expressed in g/kg body weight as means ± S.D. (n = 4).

The upper, lower and middle small intestine refer to the first 5 cm of the proximal intestine, the last 5 cm of the distal intestine, and the remaining part of small intestine.

*Significant differences from the control group at p < 0.05; one-way ANOVA with Dunnett's post hoc test.

Light microscopic observation of HE-stained sections of the small intestine showed no change of crypts or villi in any of the EN groups at 4 weeks (Fig. 1).

HE and Oil red O staining of the liver of rats fed EN for 4 weeks is shown in Figure 2. Prominent vacuolation of the liver in the digested diet group was seen in HE-stained sections (Fig. 2C). The extent of Oil red O staining was greater in the digested diet group compared with the control group (Figs. 2E and 2F), suggesting hepatic steatosis. This is not surprising, because the digested diet does not contain lipid, and it is well known that essential fatty acid deficiency leads to triglyceride deposition due to facilitation of fatty acid synthesis and inhibition of fatty acid oxidation in liver.22-24

DAO activity: Plasma DAO activity is a reflection of small intestine mucosal atrophy, and is used widely as an index of morphological change of the small-intestinal mucosa.29 We observed a significant decrease in plasma DAO activity in the semi-digested diet group at 2 and 4 weeks, but it amounted to less than 10%. There was no change of serum DAO activity in the digested diet group or the elemental diet group.

Influence of long-term EN on digoxin pharmacokinetics: Plasma digoxin concentrations after oral administration are shown in Figure 3, and the kinetic parameters are summarized in Table 2.

As shown in Figure 3, the time courses of plasma concentration of digoxin showed rather different patterns of variation among the three EN diet groups, compared to the control diet group. The plasma concentration of digoxin was significantly reduced at several time points in the semi-digested diet group compared with the control (Fig. 3B). On the other hand, the plasma concentration of digoxin in the digested diet group and elemental diet group was significantly increased at many time points compared with the control diet group (Figs. 3C and 3D).

As shown in Table 2, among various pharmacokinetic parameters evaluated, the area under the concentration curve of digoxin (AUC) was significantly reduced in the semi-digested diet group (p < 0.05), but was significantly increased in the digested diet group and elemental diet group versus the control (p < 0.05). The first-order absorption rate constant of digoxin (ka) was significantly reduced in the semi-digested diet group versus the control (p < 0.05). The elimination rate constant of digoxin (ke) was reduced in the digested diet group and elemental diet groups versus the control.

mRNA expression levels of transporters and metabolic enzyme in the small intestine, liver and kidney: The results of quantitative real-time PCR are summarized in Figures 4–7. Figure 4 shows the mRNA expression levels of influx transporters of the Slco (Oatp) family at the small intestine in each EN group at 4 weeks, and Figure 5 shows the mRNA expression of efflux transporters of the Abcb (Mdr1) family at the same time point. Figure 6 shows the mRNA expression level of Abcb1a in the kidney in each EN group at 4 weeks. Figure 7 shows the mRNA expression level of Cyp3a2 in the liver in each EN group at 4 weeks.

The Slco1a4 mRNA level was significantly reduced at the upper small-intestinal mucosa in all EN diet groups, compared with the control group (Fig. 4A, p < 0.05). However, the Slco1a4 mRNA level at the middle and lower parts of the small intestine was not affected in any of the EN diet groups. The mRNA level of Slco1a5 at the upper and middle small intestine was decreased, though not significantly, in the semi-digested diet group, while its level at the lower small intestine was significantly reduced in all EN diet groups versus the control group (Fig. 4B, p < 0.05).

The Abcb1a mRNA level was significantly increased at the upper small-intestinal mucosa in the elemental diet group versus the control group (p < 0.05), and was also increased, though not significantly, in the semi-digested diet group and digested diet group (Fig. 5A). In contrast, the Abcb1a mRNA level at the middle and lower parts of small intestine was significantly increased in the digested diet group and elemental diet group (Fig. 5A, p < 0.05). There was no significant change of Abcb1b mRNA level in any EN group versus the control group (Fig. 5B).

In the kidney, the Abcb1a mRNA level was significantly reduced in the digested diet group and elemental diet group (Fig. 6, p < 0.05). The Slco1a4, Slco1a5 and Abcb1b mRNA levels were not changed in any EN diet group versus the control (data not shown).

In the liver, the Cyp3a2 mRNA level was significantly reduced in the digested diet group and elemental diet group versus the control group (Fig. 7, p < 0.05). In the semi-digested diet group, this change was not observed. The Abcb1b mRNA level was significantly increased at the same site only in the digested diet group (data not shown). The Slco1a4 and Abcb1a mRNA levels were not changed in any EN diet group versus the control (data were not shown).

Protein expression levels of P-gp and Cyp3a2: Western blot analysis was performed to assess the effect of EN on the protein expression levels of P-gp and Cyp3a2. Specific bands were detected at around 170 kDa for P-gp (Fig. 8) and at around 54 kDa for Cyp3a2 (Fig. 9). The protein expression of P-gp was significantly increased at the upper small intestine in the elemental diet group versus the control group (p < 0.05), and was also increased, though not significantly, in the semi-digested diet group and digested diet group (Fig. 8). The protein expression of Cyp3a2 in the liver was significantly reduced in the digested diet group and elemental diet group compared with the control group (Fig. 9). In the semi-digested diet group, Cyp3a2 expression was not changed versus the control. These results are consistent with the changes in mRNA expression levels.

Influence of long-term EN on digoxin pharmacokinetics in humans: The records of 568 patients (60% males, median age 71
Fig. 1. Light micrographs of hematoxylin-eosin (HE)-stained sections of the small intestine of rats given enteral nutrition for 4 weeks

Fig. 2. Light micrographs of liver sections of rats given enteral nutrition for 4 weeks

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Among them, 547 were in the normal diet group (ingesting regular meals), and 21 were in the semi-digested diet group (ingesting semi-digested diets such as YH-Flore\textsuperscript{μ}, Inslow\textsuperscript{μ}, Oxepa\textsuperscript{μ}, Impact\textsuperscript{μ}, Lifelon\textsuperscript{μ}, Renalen-MP\textsuperscript{μ}, and Termeal\textsuperscript{μ}). As shown in Figure 10, the C/D ratio of digoxin was significantly reduced in the semi-digested diet group versus the normal diet group.

### Table 2. Pharmacokinetic parameters of digoxin after oral administration of 0.05 mg/kg of digoxin to rats given various kinds of enteral nutrition for 4 weeks

<table>
<thead>
<tr>
<th></th>
<th>Control diet</th>
<th>Semi-digested EN diet</th>
<th>Digested EN diet</th>
<th>Elemental EN diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC_{0-∞} (ng/mL·h)</td>
<td>25.0 ± 3.6</td>
<td>12.2 ± 2.8*</td>
<td>61.5 ± 17.9*</td>
<td>110.7 ± 25.7*</td>
</tr>
<tr>
<td>CL_{p} (mL/min)</td>
<td>40.7 ± 6.8</td>
<td>83.9 ± 15.0*</td>
<td>22.6 ± 8.1</td>
<td>10.6 ± 2.5</td>
</tr>
<tr>
<td>T_{max} (h)</td>
<td>0.69 ± 0.18</td>
<td>0.88 ± 0.24</td>
<td>0.63 ± 0.21</td>
<td>0.74 ± 0.17</td>
</tr>
<tr>
<td>C_{max} (ng/mL)</td>
<td>4.66 ± 0.65</td>
<td>1.95 ± 0.32*</td>
<td>8.80 ± 2.02*</td>
<td>8.05 ± 0.93*</td>
</tr>
<tr>
<td>k_{c} (h^{-1})</td>
<td>6.08 ± 0.81</td>
<td>3.31 ± 0.77*</td>
<td>4.89 ± 0.84</td>
<td>4.70 ± 1.09</td>
</tr>
<tr>
<td>k_{e} (h^{-1})</td>
<td>0.53 ± 0.15</td>
<td>0.49 ± 0.18</td>
<td>0.37 ± 0.07</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>V_{d} (L/kg)</td>
<td>14.4 ± 2.7</td>
<td>22.5 ± 4.8</td>
<td>14.7 ± 8.2</td>
<td>7.5 ± 1.1</td>
</tr>
</tbody>
</table>

Values are expressed as means ± S.E. (n = 6–9). *Significant differences from the control group at p < 0.05; one-way ANOVA with Dunnett’s post hoc test.
In order to confirm the suitability of our rat models for examining the effects of long-term EN with different types of diets on digoxin pharmacokinetics, we initially examined the effects of long-term EN with different types of diets on body weight and tissue weights. In order to avoid the effects of stress, the diets were freely administered from water bottles. All groups showed good growth. The mean body weight in the semi-digested diet group was significantly increased compared with the control group at \( p < 0.05 \); one-way ANOVA with Dunnet’s post hoc test.

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**Discussion**

In order to confirm the suitability of our rat models for examining the effects of long-term EN with different types of diets on digoxin pharmacokinetics, we initially examined the effects of long-term EN with different types of diets on body weight and tissue weights. In order to avoid the effects of stress, the diets were freely administered from water bottles. All groups showed good growth. The mean body weight in the semi-digested diet group was significantly increased compared with the control group at \( p < 0.05 \), but the reason for this was considered to be that the nutrient concentration of Termeal is higher than that of other EN diets. We found that the small intestine weight was significantly increased in the semi-digested diet group, and this result is in agreement with previous reports that feeding of elemental diet reduces the small-intestinal mucosa weight. However, no atrophic change of the small-intestinal mucosa was seen in light microscopic observation of HE-stained sections. There was a small but significant decrease of serum DAO activity in the semi-digested diet group, but not in the other EN diet groups. Severe atrophic damage to the small-intestinal mucosa is associated with a much larger decrease of serum DAO activity. Thus, these results suggest that the small-intestinal mucosal function might have been well maintained during long-term EN. Based on these results, we judged that these long-term EN model rats were appropriate for our investigation.

The effects of long-term EN on digoxin pharmacokinetics were different in each of the EN diet groups. Digoxin is well absorbed after oral administration, mainly in the proximal part of the small intestine, and is mostly eliminated unchanged by renal excretion. Contrary to earlier suggestions that intestinal absorption of digoxin occurs by passive diffusion, it has recently been shown that active transport by members of the organic anion transporting polypeptide (Oatp) family is involved in digoxin absorption. Digoxin is also a substrate of the efflux transporter P-glycoprotein (P-gp). On the other hand, in rats, about 70% of digoxin is metabolized by Cyp3a2 in the liver. Therefore, we investigated the influence of long-term EN on digoxin pharmacokinetics, and the relevance of active transport at the small-intestinal mucosa, metabolism in the liver and excretion in the kidney by measuring changes in the expression of transporter and metabolic enzyme genes, using quantitative real-time PCR.

We administered digoxin to rats without fasting in order to reproduce the clinical situation. It has been reported that food-drug interaction has little effect on digoxin absorption or bioavailability in the postprandial state, although \( t_{max} \) of digoxin was delayed slightly. On the other hand, a fiber-rich diet was reported to inhibit digoxin absorption and to reduce plasma digoxin concentration in humans. However, the digested and elemental diets used in this study do not contain fiber, and the control and semi-digested diets are not fiber-rich (0.5 g/100 kcal and 0.3 g/100 kcal, respectively). So, we considered that food-drug interaction involving fiber and other nutrients would not have been significant in the EN diet groups.

Compared with the control group, the mRNA expression of Slc01a4 at the upper small intestine was significantly reduced in all EN diet groups. In contrast, the mRNA expression of Abcb1a (Mdr1) and protein expression of P-gp in the upper small intestine were significantly increased in the elemental diet group, and were also increased, though not significantly, in the semi-digested diet group and digested diet group. Digoxin absorption occurs predominantly at the proximal part of the small intestine, so changes of Slco (Oatp) family influx transporter and Abc (Mdr) family efflux transporter expression levels at the upper small intestine are most likely to influence digoxin absorption kinetics.

The plasma concentration of digoxin was significantly reduced.
at several time points after digoxin administration in the semi-digested diet group, compared with the control. The pharmacokinetic analysis exhibited a marked and significant decrease of \( k_e \) and AUC in this diet group, and this is consistent with the reduced expression of Slco influx transporter (Oatp) and the increased expression of Abc efflux transporter (P-gp) at the upper small intestine in this diet group. Thus, absorption of digoxin appears to be reduced as a result of reduced expression of uptake transporter and increased expression of efflux transporter in the semi-digested diet group.

The plasma digoxin concentration was significantly increased at many time points after digoxin oral administration in the digested diet group and elemental diet group compared with the control diet group. Moreover, AUC was significantly increased in both groups versus the control. There was a tendency for decrease of \( k_e \) in the semi-digested diet group, but there was also a tendency for decrease of \( k_e \) in both groups. In contrast to the semi-digested group, these results seem not to be consistent with the changes in transporter expression at the upper small intestine in the digested diet group and elemental diet group. The mRNA expression levels of Cyp3a2 in the liver and Abcb1a in the kidney were significantly reduced in both groups compared with the control group. On the other hand, these changes were not observed in the semi-digested diet group. The reduction of Abcb1a mRNA expression in the kidney may lead to a reduction of renal excretion of digoxin in the digested diet group and elemental diet group versus the control. In rats, digoxin is metabolized to the extent of about 70% in the liver, so the change of Cyp3a2 expression in the liver is likely to influence digoxin metabolism. The protein expression of Cyp3a2 in the liver was also significantly reduced in the digested diet group and elemental diet group versus the control. Thus, elimination of digoxin appears to be reduced as a result of reduced expression of Cyp3a2 in the liver and efflux transporter in the kidney in the digested diet group and elemental diet group.

In our study, the digested diet induced hepatic steatosis and reduced protein and mRNA expression levels of Cyp3a2 in the liver. Moreover, there was a decreasing tendency in the elimination rate constant (\( k_e \)) of digoxin in the rats fed with digested diet. These results strongly indicate that Cyp3a2 activity was reduced. It has already been reported that fat-free TPN induced hepatic steatosis and reduced mRNA expression level of Cyp3a2.\(^{41}\) Moreover, CYP3A4 activity and mRNA expression level were reduced about 50% in steatotic livers compared with nonsteatotic livers in humans.\(^{42}\) A decreasing trend in CYP3A4 activity was also reported in the livers of nonalcoholic fatty liver disease patients.\(^{43}\) All of these observations support the idea that the hepatic steatosis induced by the digested diet was associated with reduced Cyp3a2 activity. The elemental diet did not induce hepatic steatosis, but it would induce essential fatty acid deficiency because the elemental EN diet contains little fat. The essential fatty acid deficiency may reduce Cyp3a2 activity as well as hepatic steatosis.

However, we cannot rule out the possibility that the nitrogen source of the digested and elemental diets might decrease the expression of Cyp3a2 in the liver. The nitrogen source might also increase Mdr1a in the middle and lower part of the small intestine and the kidney. To our knowledge, there is no report indicating that different nitrogen sources influence metabolic enzyme or transporter expression levels. Further studies, for example, to clarify the effect of nutrient composition on relevant gene transcription factors, might be of interest.

The changes in plasma concentration and pharmacokinetic parameters of digoxin in the EN diet groups were suggestive of different patterns of change in response to the different EN diets. Our results indicate that the absorption capacity of digoxin is reduced in all EN diet groups, and the metabolism and excretion capacities are reduced in the digested diet group and elemental diet group.

In humans, the influence of long-term EN with semi-digested diet on the pharmacokinetics of digoxin was similar to that in rats. The C/D ratio of digoxin was significantly reduced in the semi-digested diet group, compared with the normal diet group. Although digoxin is mostly eliminated by renal excretion, \( k_e \) showed no difference between the two groups in rats. If this is also the case in humans, the reduction of the C/D ratio of digoxin in the semi-digested diet group may be a consequence of changes in processes other than metabolism and excretion. These results raise the possibility that the influence of long-term EN with semi-digested diet on the pharmacokinetics of digoxin involves a change in the absorption process of digoxin, taking the result in rats into consideration. The absorption of digoxin may be decreased as a result of reduced expression of uptake transporters and increased expression of efflux transporter induced by the semi-digested diet in humans, as well as rats.

Although a fiber-rich diet was reported to inhibit digoxin absorption, there has been no report on the effect of insufficient fiber content on expression of any transporter or drug-metabolic enzyme. It is recommended that healthy adults take more than 18 g fiber per day. Assuming that a man takes 2,000–2,500 kcal per day, this value corresponds to 0.7–0.9 g fiber/100 kcal, and normal rat chow contains 0.8 g fiber/100 kcal. The control diet used in this study (Calorie Mate\(^{45}\)) contained 0.5 g fiber/100 kcal. The digested and elemental diets include no fiber. Although the semi-digested diet includes 0.3 g fiber/100 kcal, this is much less than the required amount. In the present study, the expression levels of Slco1a4 and Slco1a5 were decreased by the semi-digested diet, as well as the other two ENs, compared with the control diet. Moreover, the digoxin pharmacokinetics was altered in rats and humans. As the nutrient compositions are rather similar between the normal diet and semi-digested diets, the difference in fiber content might be sufficient to affect the expression of Slco transporters and thus the digoxin pharmacokinetics.

In humans, digoxin is mainly excreted unchanged into the urine, whereas about 70% of digoxin is eliminated by hepatic metabolism by Cyp3a2 in rats. In spite of this species difference, blood digoxin level was decreased by the semi-digested diet both in rats and humans. Unfortunately, none of our digoxin-treated patients was receiving digested or elemental diet, so we are unable to compare the effects of these diets between rats and humans. In rats, the semi-digested diet decreased the intestinal expression level of Slco1a4 (Fig. 4) and increased intestinal P-gp (Figs. 5 and 8), but did not affect hepatic Cyp3a2 (Figs. 7 and 9) or renal Abcb1a (Fig. 6). It is known that there are significant species differences in OATP. Indeed, although digoxin is transported by rat Oatp1a4, which is expressed in the small intestine, human intestinal OATP2B1 does not accept digoxin as a substrate.\(^{46}\) Although digoxin is subject to efflux transport by P-gp, the intestinal transporter responsible for the luminal uptake in the small intestine remains to be identified.

Our findings suggest that long-term EN alters active drug transport processes in the small intestine, metabolic activity in
the liver and excretion in the kidney in ways that are dependent upon the EN diet composition, and these changes may affect the pharmacokinetic characteristics of digoxin.

In conclusion, Long-term EN diets altered the expression levels of digoxin influx and efflux transporters in the small intestine and efflux transporters in the kidney, as well as metabolic activity in the liver in ways that depended on the EN diet composition. As these changes appear to affect the pharmacokinetic characteristics of digoxin, further investigation appears warranted to examine the effects of long-term EN on the pharmacokinetics of other clinically important drugs.

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References