Acidic Xylooligosaccharide promotes recovery from iron deficiency anemia by enhancing serum iron level in rats

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Abstract

Iron deficiency anemia (IDA) is one of the most serious forms of malnutrition. A promotion function of mineral absorption has been reported for neutral oligosaccharides, but not acidic xylooligosaccharide (U-XOS), which is a novel oligosaccharide. We hypothesized that U-XOS could promote recovery from IDA by enhancing the serum iron level. Therefore, the aim of this study was to investigate whether or not U-XOS was useful for recovery from IDA in the rat. Twenty-four female Sprague-Dawley rats were randomly divided into a control group fed a control diet (4.0 mg Fe /100 g) and an IDA model group created by employing a low-iron diet (0.4 mg Fe /100 g). The IDA group was further divided into three subgroups on day 21: U-XOS-supplemented diet (LI-X, 4.0 mg Fe /100 g), low-iron diet (LI), and control diet (LI-C) groups. No significant differences in the serum iron transferrin saturation levels were demonstrated between the control and LI-X groups on days 26 and 35. The divalent metal transporter 1 and ferroportin mRNA expression levels shown in the first segment of the small intestines showed a significant decrease in the LI-X group, compared with the LI group. A significant decrease in the hepatic hepcidin mRNA expression level and iron content was also demonstrated in the LI-X group, compared with the control group, but not compared with the LI group. These results suggested that U-XOS could promote recovery from IDA by enhancing serum iron at an early stage of the recovery process.

Introduction

It is well known that iron deficiency anemia (IDA) is one of the most serious forms of malnutrition. According to the World Health Organization, it is estimated that more than 1.6 billion people are suffering from anemia, and approximately half of these people have IDA [1], showing that IDA has been increasing worldwide.

In order to achieve any improvement in subjects with IDA, dietary management is most important in order to increase the dietary iron intake. However, there are three major factors that affect iron absorption. The first factor is the form of dietary iron. Most dietary iron is in the form of Fe^{3+}, and is usually reduced to Fe^{2+} by the Fe-reduction factor and imported into the mucosal cells by the divalent metal transporter 1 (DMT1), which is one of the iron transporters in the small intestines [2]. The second factor is the storage iron in the body. If serum iron levels and iron stores are decreased, secretion of the hepcidin from

the liver decreases, and then the expression of iron transporter ferroportin, which exports Fe^{2+} from cells on the basolateral membrane, increases. As a result, the amount of iron absorption increases [3-6]. The third factor is the consumption of dietary components. Several food factors have been demonstrated to enhance iron absorption, for example, ascorbic acid [7-9], cysteine-containing peptides [10-12], phosphopeptide [13-15], sugar alcohol [16, 17], polysaccharides [18, 19], and so on. However, relatively little attention has been paid to the effects of the food matrix on iron absorption.

Recently, it was reported that indigestible oligosaccharides, such as fructooligosaccharide [20, 21], difructose anhydride III [22-24], and xylooligosaccharide [25], promote mineral absorption. Acidic xylooligosaccharide (U-XOS) is a novel oligosaccharide in which glucronic acid is linked to xylooligosaccharide by α-1, 2 bonds [26]. U-XOS has been reported to have an antibacterial effect, inhibitory effects on stress-induced gastric inflammation, preventive effects on contact hypersensitivity, and preven-

tive effects on the development of atopic dermatitis [27, 28]. However, the promotive effects of U-XOS on serum iron and recovery from IDA have not been examined to date.

Therefore, we hypothesized that U-XOS could promote recovery from IDA by enhancing the serum iron level. Accordingly, the aim of this study was to investigate whether or not U-XOS was useful for recovery from IDA in the rat.

Methods and Materials

The structure and composition of Acidic xylooligosaccharide (U-XOS)

The structure of U-XOS is shown in Figure 1. U-XOS contains one or more 4-*O*-methyl glucuronic acids as side chains and it is a xylo-oligomer with an average of 10 polymerization units. U-XOS is prepared from hardwood kraft pulp, such as eucalyptus, according to the method described by Izumi *et al.* [29]. The U-XOS employed in this study was kindly donated by Oji Paper (Tokyo, Japan). The composition of U-XOS was shown in Table 1 [26].

Table 1. *Composition of experimental diet*

Ingredient	Amount (g/100g diet)		
Casein	20.0		
α-starch	45.7		
Sucrose	22.8		
Mixed oil[a]	5.0		
Vitamin mixture[b]	1.0		
Mineral mixture[c]	3.5		
	Control diet	**U-XOS diet**	**Low iron diet**
Cellulose	2.0	-	2.0
U-XOS[d]	-	2.0	-
Iron (III) Citrate	180.0	180.0	-
Iron content	0.0040	0.0040	0.0004

a. Rapeseed oil /soybean oil ratio = 7/3.
b. AIN-76 vitamin mixture (per g): vitamin A, 400 IU; vitamin D_3, 100 IU; vitamin E, 5 mg; vitamin K_3, 0.005 mg; vitamin B_1, 0.6 mg; vitamin B_2, 0.6 mg; vitamin B_6, 0.7 mg; vitamin B_{12}, 0.001 mg; D-biotin, 0.02 mg; folic acid, 0.2 mg; calcium pantothenate, 1.6 mg; nicotinic acid, 3 mg; choline chloride, 200 mg; sucrose, 0.968 g.
c. AIN-76 mineral mixture (g/kg mixture): calcium phosphate dibasic, 500.0; sodium chloride, 74.0; potassium citrate, 220.0; potassium sulfate, 52.0; magnesium oxide, 24.0; manganese carbonate, 3.5; zinc carbonate, 1.6; cupric carbonate, 0.3; potassium iodate, 0.01; sodium selenite, 0.0066; chromium potassium sulfate, 0.55; sucrose, 124.03.
d. U-XOS (g/kg U-XOS): water content, 28; protein content, less than 1; lipid content, less than 1; U-XOS, 972 (carbohydrate content, 901; ash content derived from the sodium salt of uronic acids, 71). Monosaccharide composition of U-XOS from hardwood kraft pulp (%): xylose, 81.0; galactose, 4.4; glucose, traces; uronic acid, 14.6.

Study design

This study was conducted in accordance with the guidelines for animal experimentation published by Kyoto Prefectural University. Twenty-four female Sprague-Dawley rats aged 4 weeks (Japan SLC, Hamamatsu, Japan) were employed in the study. The rats were individually housed in stainless steel cages at a controlled temperature of 22 – 24°C, a relative humidity of 40 – 60%, and a light cycle of 12 hours with free access to distilled water (the iron content of the distilled water had been measured). The composition of the diets used in the experiment was as shown in Table 1. The rats were randomly divided into two groups. For the first group (C group, n = 6, weighing 106 – 112 g), a control diet was supplied for 35 days. The second group (base LI group, n = 18, weighing 94 – 121 g), fed a low-iron diet for 21 days, was employed to create IDA rats. IDA rats were then divided three subgroups on the basis of weight and hemoglobin concentration. Each subgroup was fed either a low-iron (LI) diet (LI group, n = 6, weighing 155 – 195 g), a U-XOS– supplemented diet (LI-X group, n = 6, weighing 153 – 177 g), or the control diet (LI-C group, n = 6, weighing 163 – 182 g) for another 14 days. The LI-C and LI-X groups were provided with the diet on the following day, with the same amount of diet freely provided to the LI group during the pair-feeding period. All diets were prepared according to the AIN-76 formulation with one modification (addition of choline chloride). The LI diet contained 0.4 mg Fe /100 g without any ferrous citrate in the mineral mixture: U-XOS was added to 2% of the whole diet by substituting for cellulose. Blood samples, all 280 μL, were drawn from the tail vein of all of the animals every 4 days during the last 14 days of the study. Body weight and food intake were recorded at the same time everyday. The rats were euthanized during the early phase of the light cycle in a non-fasting state, by cervical dislocation under ether anesthesia, and blood samples drawn from the inferior vena cava were collected in tubes with heparin. Samples of the liver and the small intestinal mucosa (upper side, 1/4th) were also collected.

Blood constituent analysis

Hemoglobin concentration was measured using Hemoglobin B-test Wako (Wako Pure Chemical Industries, Osaka, Japan). The hematocrit level was measured after centrifugation of the blood at 12,000 rpm for 5 minutes at 4°C. Serum iron levels and unsaturated iron binding capacity were measured using Detaminer Fe and UIBC (Kyowa Medix, Tokyo, Japan) with an automatic biochemical analyzer (CL-8000, Shimadzu, Kyoto, Japan).

Total iron binding capacity (TIBC) and serum transferrin saturation were calculated as follows:

TIBC = serum iron + UIBC

Serum transferrin saturation = serum iron / TIBC ×100.

Estimation of gene expression

Total RNA was isolated from the homogenized mucosa and liver samples using Total RNA Isolation mini kit (Agilent Technologies, California, USA). The cDNA was synthesized from isolated RNA using ReverTra Ace (Toyobo, Osaka, Japan), according to the manufacturer's instructions. A real-time polymerase chain reaction (PCR) for gene expression analysis was performed using DNA Engine Opticon and Opticon Monitor software (Bio-Rad Laboratories, California, USA). TaqMan primer pairs /probes for gene analysis were obtained using TaqMan Gene Expression Assay (Applied Biosystems, California, USA): Rn00565927_m1: DMT1, Rn00591187_m1: Ferroportin, Rn00584987_m1: Hepcidin, Rn00667869_m1: β-actin. Reactions were performed with 10 μL of Premix EX Taq (Takara Bio, Ohtsu, Japan), 1 μL of the primer pairs /probes sets and 3 μL of cDNA in a final volume of 20 μL. After heating the test sample at 96°C for 10 seconds, 50 PCR cycles were performed as follows: 95°C for 7 seconds, 60°C for 30 seconds and 72°C for 20 seconds. DMT1, ferroportin, and hepcidin mRNA were normalized against the mRNA expression of the housekeeping gene β-actin.

Iron content of hepatic tissue

All liver samples were perfused with saline, and weighed (0.5g), and treated by the wet-ash method using a microwave extraction system (Ethos-1000, Milestone, Italy). The samples were diluted to 10 mL with 0.5% hydrochloric acid solution after evaporation to dryness. After suitable dilution, iron concentrations of the samples were measured by flame Zeeman-effect atomic absorption spectrometry (Z-6100, Hitachi, Tokyo, Japan) using a standard solution (Wako Pure Chemical Industries, Osaka, Japan). We determined that the coefficient of variation was 0.04. Iron concentrations were expressed on a wet-weight basis.

Statistical analyses

Data (C, LI, LI-X and LI-C group, n = 6; base LI group, n = 18) were presented as means ± standard error (SEM). Before assessing the different variables, we carried out a Bartlett test to check the normal distribution of the variables. Data that fit the normal distribution were compared by 1-way analysis of variance (ANOVA) followed by the Tukey-Kramer test (Figs. 2 – 4; all parameters on day 35, Table 2), or Student's *t* test (all parameters on day 21, Table 2). The level of significance was set at $P < 0.05$.

Table 2. *Body weight gain, food intake and blood parameters on day 21 and day 35 after the start of study*

	day 21		day 35			
	base LI	**C**	**LI**	**LI-X**	**LI-C**	**C**
Body weight gain (g/day)	3.0 ± 0.1	3.0 ± 0.2	1.4 ± 0.2	1.3 ± 0.1	1.1 ± 0.0	1.5 ± 0.2
Food intake (g/day)	11.7 ± 0.1	11.8 ± 0.4	11.5 ± 0.6	11.5 ± 0.0	11.5 ± 0.0	11.9 ± 0.6
Hematocrit level (%)	33.0 ± 0.7[a]	47.1 ± 1.2[b]	28.5 ± 1.4[a]	45.1 ± 1.5[b]	47.7 ± 2.0[b]	49.6 ± 0.9[b]
Hemoglobin concentration (g/dl)	9.7 ± 0.3[a]	15.4 ± 0.7[b]	8.1 ± 0.6[a]	15.4 ± 1.0[b]	15.3 ± 0.6[b]	17.8 ± 0.6[b]
Total iron binding capacity* (μg/dl)	878 ± 11[a]	539 ± 45[b]	934 ± 16[a]	711 ± 19[b]	691 ± 26[bc]	615 ± 14[c]
Unsaturated iron binding capacity (μg/dl)	848 ± 12[a]	295 ± 71[b]	909 ± 17[a]	491 ± 89[bc]	541 ± 44[c]	309 ± 34[b]

*Normal rats fed low iron diet (base LI: n = 18) for 21 days or control diet (C: n = 6) for 35 days. Rats in the base LI group divided three subgroups, were fed either a low iron diet (LI: n = 6), a U-XOS-supplemented diet (LI-X: n = 6) or the control diet (LI-C: n = 6) for another 14 days. Differences were considered significant when P < 0.05. *Total iron binding capacity = Serum iron + Unsaturated iron binding capacity.*

Results

Effect of U-XOS on recovery from IDA

Body weight gain, food intake and blood parameters on day 21 and day 35 after the start of study are shown in Table 2. No significant difference in body weight gain and

food intake was observed between the two base groups on day 21 or among the four subgroups on day 35. On day 21, the hematocrit level and hemoglobin concentration of the base LI group (LI, LI-X and LI-C group) were lower than those of the C group, and the total iron binding capacity and unsaturated iron binding capacity of the base LI group increased compared with the C group, confirming

that the LI group had developed IDA. On day 35, the hematocrit level and the hemoglobin concentration of the LI-X group were higher than those of the LI group, but no significant difference was demonstrated between the C and LI-C groups, indicating that the LI-X and LI-C groups showed recovery from IDA. The total iron binding capacity of the LI-X group decreased remarkably compared with that of the LI group and increased compared with the C group, and both differences were statistically significant. The unsaturated iron binding capacity of LI-X group decreased significantly compared with the LI group and increased compared the C group, but no statistical difference was shown between the LI-X and C groups.

Figure 2 shows the changes in the serum iron and transferrin saturation level of the groups on day 21, 26, and 35 after the start of the study. On day 21, the serum iron and transferrin saturation level of the base LI group (LI, LI-X and LI-C groups) were significantly lower than those of the C group, confirming that the base LI group had developed an iron deficiency state. The serum iron level of the LI-X group drastically increased compared with that of the LI and LI-C groups, but no significant difference was demonstrated compared with the C group on day 26. On day 35, the serum iron level of the LI-X group had been maintained at the same level as that on day 26. Showing a pattern similar to the serum iron level, the serum transferrin saturation level of the LI-X group markedly increased compared with the LI and LI-C groups on day 26, but it was comparable with the C group on day 26 and 35.

Expression of transporter mRNA

Figure 3 shows the iron transporter DMT1 and ferroportin mRNA expression level in first segment of the small intestines on day 35 after the start of the study. The DMT1 mRNA expression levels of the LI-X group were significantly lower than those of the LI group, but no statistical difference was shown between the LI-X and the other groups. The ferroportin mRNA expression level of the LI-X group was remarkably decreased compared with that of the LI group, and was lower than that of the LI-C group. However, these decreases were not statistically significant. No significant difference was shown in the ferroportin mRNA expression level between the LI-X and C groups.

Iron content and hepcidin mRNA in the liver

Iron content and hepcidin mRNA expression levels in the liver on day 35 after the start of the study are shown in Figure 4. On day 35 after the start of the study, the LI-X group showed a remarkable and statistically significant decrease in hepatic iron content compared with the C group, but no statistically significant difference was shown between the LI-X and the other groups. The hepatic hepcidin mRNA expression levels of the LI-X group decreased significantly compared with those of the C

group, but no statistically significant difference was shown between the LI-X and the other groups.
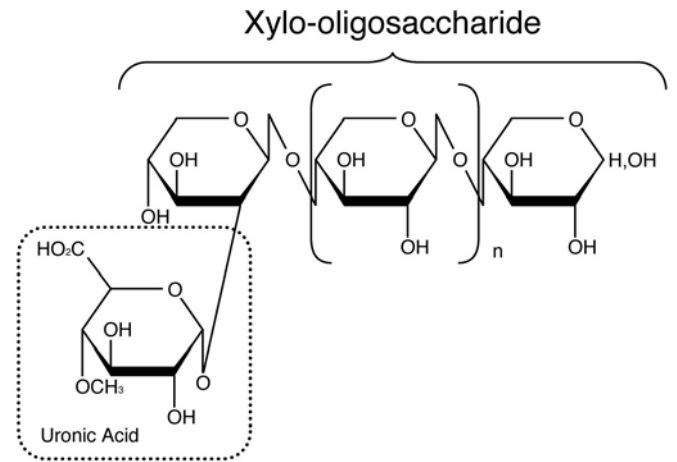

Figure 1. *The structure of acdic xylo-oligosaccharide (U-XOS)*
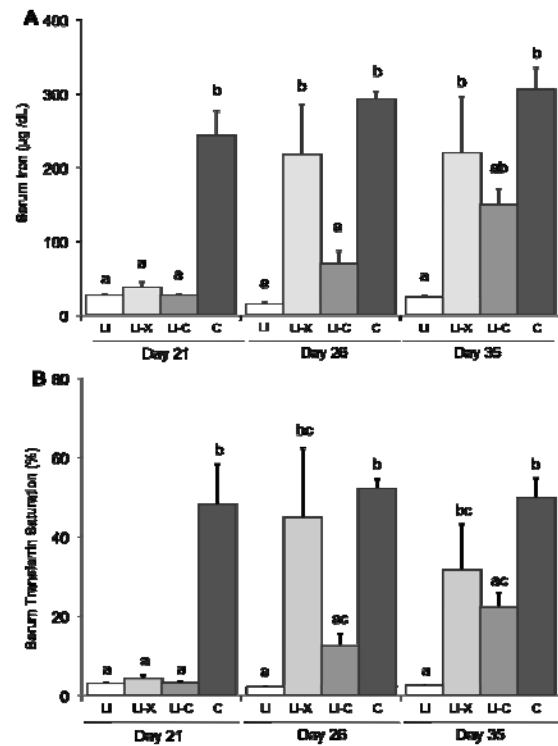
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Figure 2. *Serum iron (A) and Serum transferrin saturation (B) on the day 21, 26, and 35 after the start of the study. Iron deficiency anemia rats, fed low iron diet for 21 days, and divided into three subgroups, were fed either a low iron diet (LI: n = 6), a U-XOS-supplemented diet (LI-X: n = 6) or the control diet (LI-C: n = 6) for another 14 days. Nomal rats were fed the control diet (C: n = 6) for 35 days. Values are represented as means + SE. Values with an unlike letter were significant: P < 0.05.*
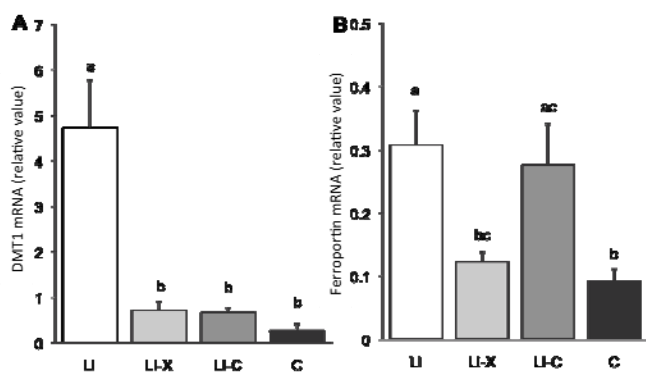
Figure 3. *Iron transporter DMT1 (A) and Ferroportin (B) mRNA expression in the first segment of small intestines (upper side, 1/4th) on day 35 after the start of the study. Iron deficiency anemic rats, fed a low iron diet for 21 days, and divided into three subgroups, were fed either a low iron diet (LI: n = 6), a U-XOS-supplemented diet (LI-X: n = 6) or the control diet (LI-C: n = 6) for another 14 days. Nomal rats were fed the control diet (C: n = 6) for 35 days. Values are represented as means + SE. Values with an unlike letter were significant: P < 0.05*
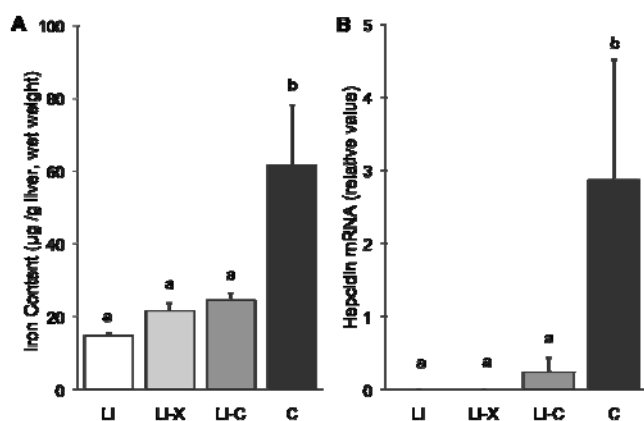

Figure 4. *Iron content (A) and hepcidin mRNA expression (B) in the liver on day 35 after the start of the study. Iron deficiency anemic rats, fed a low iron diet for 21 days, and divided into three subgroups, were fed either a low iron diet (LI: n = 6), a U-XOS-supplemented diet (LI-X: n = 6), or the control diet (LI-C: n = 6) for another 14 days. Nomal rats were fed the control diet (C: n = 6) for 35 days. Values are represented as means + SE. Values with an unlike letter were significant: P < 0.05.*

Discussion

In the present study, we investigated whether or not U-XOS was useful in achieving recovery from IDA using a rat model. Our results demonstrated that U-XOS could promote recovery from IDA by enhancing serum iron and transferrin saturation levels at an early stage of the recov-

ery process. It is necessary for dietary iron to be reduced from Fe^{3+} to Fe^{2+} ions [30, 31]. Therefore, it appeared that soluble Fe^{2+} ions in the small intestines were increased in quantity by U-XOS. In regard to the mechanisms by which U-XOS dissolves and reduces dietary iron, this may be related to the intermolecular force between U-XOS molecules, which may vary according to the number of uronic acid groups per chain, or related to the average sugar unit of U-XOS.

On the other hand, it has been reported that DMT1 expression is dramatically induced in IDA [32]. These findings suggested that the reason the DMT1 mRNA expression level resulting from the ingestion of U-XOS showed no significant difference, compared with the normal controls, was because the DMT1 mRNA expression was regulated by recovery from IDA at the end of the experiment period. However, DMT1 mRNA expression might increase at the stage where there is an observed rise in the serum iron level due to U-XOS ingestion. On the other hand, the diet with no U-XOS showed that the capability to export iron ions from the epithelial cells by ferroportin was still active at the end of the experiment.

In this study, a decrease in hepatic hepcidin and intestinal ferroportin mRNA expression was shown on day 35 in rats fed the U-XOS diet. The level of hepatic hepcidin mRNA expression decreases in response to iron deficiency [33, 34]. In the present study, the level of hepatic iron concentration and hepcidin mRNA expression significantly decreased in the LI group compared with control group on day 35. The level of hepatic iron level and hepcidin mRNA did not increase in the LI-X group compared with the control group on day 35. However, the serum iron level of LI-X group increased to almost same level of the control group on day 26 and 35. Moreover, the ferroportin mRNA level of the LI-X group was reduced to almost the same level as the control group on day 35. There is the possibility that the regulation of ferroportin mRNA expression under conditions of iron deficiency without anemia will be influenced not only by hepcidin, but also by the serum iron or transferrin saturation levels. Iron deficiency without anemia is caused primarily by asiderosis. Despite the status of the iron deficiency, this regulation of hepcidin and ferroportin mRNA expression is disadvantageous for an early recovery from asiderosis. The fact that the treatment of asiderosis requires a long-term administration of iron may be the result of such adverse regulation.

The limitations of the current study include the fact that we did not directly examine or measure the iron absorption rate. In order to elucidate the effects of U-XOS on the promotion of iron absorption, further studies should be conducted to investigate the iron balance. In addition, the IDA rats did not show any recovery of storage iron, although they were administered a diet containing iron for

14 days. However, it should be possible to examine the effect on storage iron with a U-XOS diet by lengthening the experimental period. Furthermore, from the viewpoint of preventing IDA, we should also examine the efficacy of U-XOS in regard to the difference in the amount of iron intake. Moreover, another limitation of this study was that we did not assay the short chain fatty acids and the organic acid levels. Indigestible oligosaccharides such as U-XOS reach the large intestine without undergoing enzymatic degradation, are fermented by intestinal bacteria [35], and produce the short chain fatty acid and the organic acid [36]. It has been suggested that the bacterial generation of short-chain fatty acids from oligosaccharides increases iron bioavailability [22, 37]. U-XOS is also a prebiotic [28, 38]. Therefore, one of the items on our agenda for future studies is to investigate the involvement of U-XOS in iron bioavailability.

In conclusion, we demonstrated that U-XOS could be employed to promote recovery from anemia by rapidly increasing the serum iron and transferrin saturation levels. The use of U-XOS is likely to provide important clues for dietetic treatment, in combination with dietary carbohydrates, which will be useful for promoting recovery from IDA.

Acknowledgments

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