Acidic Xylooligosaccharide Preserves Hepatic Iron Storage Level in Adult Female Rats Fed a Low-Iron Diet

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Summary Iron deficiency anemia (IDA) is one of the most serious forms of malnutrition. This experiment was conducted to investigate whether acidic xylooligosaccharide (U-XOS), expected to have a high iron bioavailability, was useful in the prevention of iron deficiency. Experiment 1: Nineteen female Sprague-Dawley rats (20 wk old) were fed three different diets for 28 d; a U-XOS-supplemented low-iron diet (LI-X, n=7), a low-iron diet (LI, n=6), and a control diet (C, n=6). On day 28, the LI-X and LI groups showed iron deficiency without anemia. A significant difference in the total and unsaturated iron binding capacity, and serum transferrin saturation level was shown in the LI-X and LI groups, compared with the C group. However, the decrease of hepatic iron content of the LI-X group was suppressed compared with the LI group. Experiment 2: Eleven male Sprague-Dawley rats (7 wk old) were fed a U-XOS-supplemented diet (X, n=5) or a control diet (C, n=6) for 7 d. No significant difference in body weight gain or food intake was demonstrated between the two groups; the apparent iron absorption rate of the X group increased clearly compared with that of the C group. These results suggested that a U-XOS diet could preserve storage of hepatic iron in adult female rats fed a low-iron diet and could prevent IDA by promotion of dietary iron absorption, inhibition of iron excretion, and/or improvement of iron bioavailability.

Key Words xyloooligosaccharide, iron deficiency anemia, iron absorption, prebiotics, anemia prevention

Iron deficiency anemia (IDA) is one of the most serious forms of malnutrition, and the prevalence of IDA has been increasing widely all over the world. 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). According to a nutrition survey carried out by the Ministry of Health, Labour, and Welfare in 2007 in Japan, the level of hemoglobin was less than 12 g/dL for about 20% of Japanese women (2), showing that Japan is certainly not an exception to these trends. It is well known that IDA develops due to a negative iron balance. Because the human body is not equipped with a positive function for iron excretion, the major cause of any iron imbalance is an insufficient iron intake. Most dietary iron is in the form of Fe2+, and is usually reduced to Fe2+ by Fe-reduction factors and imported into the mucosal cells by divalent metal transporter 1 (DMT1), which is one of the iron transporters in the small intestines (3). Because Fe2+ is absorbed more efficiently than Fe3+ by DMT1 (4), soluble Fe2+ ion is very important for the absorption of dietary iron. However, the dietary iron absorption rate fluctuates widely according to the amount of iron in the body and/or the consumption of dietary components that promote or inhibit iron absorption (5–7). When serum iron levels are low and iron stores decrease, secretion of hepcidin from the liver decreases, and the expression of the iron transporter ferroportin, which exports Fe2+ from the basolateral membrane of enterocytes, increases. As a result, the amount of iron absorption increases. In contrast, when the storage requirements for iron have been fulfilled, hepatic hepcidin expression increases compared with the iron deficiency, and ferroportin expression decreases in the enterocytes, controlling excessive iron absorption (8–10). If dietary components with an increased soluble Fe2+ ion content exist in the intestinal tract, it should supply iron to the body under iron deficiency conditions, and avoid excessive iron absorption by homeostasis under iron sufficiency conditions.

Several food factors have been demonstrated to enhance iron bioavailability, for example, ascorbic acid (11), cysteine-containing peptides (12), phosphopeptide (13), sugar alcohol (14), and polysaccharides (15). Moreover, it has been reported that indigestible oligosaccharides, such as fructooligosaccharide (16), difructose anhydride III (17), and xylooligosaccharide (18), promote mineral absorption. We examined the effects of acidic xylooligosaccharide (U-XOS), one of the functional indigestible oligosaccharides, on recovery

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If amelioration of the iron biological utilization due to U-XOS is demonstrated, U-XOS is employed as a safe food component preventing IDA. In the present study, from the viewpoint of preventing IDA, we examined the usefulness of U-XOS in adult female rats fed a low-iron diet.

MATERIALS AND METHODS

Acidic xylooligosaccharide (U-XOS). The structure of U-XOS is shown in Fig. 1. U-XOS contains one or more 4-O-methyl glucuronic acids as side chains and it is a xylooligomer with an average of 8–10 polymerization units. The U-XOS employed in this study was prepared from hardwood kraft pulp, such as eucalyptus, according to the method described by Izumi et al. (20). The U-XOS employed in this study was kindly donated by Oji Paper (Tokyo, Japan). The composition of U-XOS is shown in Table 1 (21).

Animal experimental protocol. This study was performed in accordance with the guidelines for animal experimentation at Kyoto Prefectural University. Nineteen female Sprague-Dawley rats aged 20 wk (experiment 1) and 11 male Sprague-Dawley rats aged 7 wk (experiment 2) were employed in the study (Japan SLC, Inc., Hamamatsu, Japan). 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 h with free access to distilled water (the iron content of the distilled water had been measured). Body weight and food intake were recorded at the same time every day. The compositions of the diets used in the experiments were as shown in Table 1. All diets were prepared according to the AIN-76 formulation with one modification (addition of choline chloride).

In experiment 1, the rats were divided into three groups on the basis of body weight. Each group was fed a low-iron diet (LI group, n=6, body weight: 274.1±4.3 g), a U-XOS-supplemented low-iron diet (LI-X group, n=7, body weight: 266.3±2.9 g) or a control diet (C group, n=6, body weight: 273.3±5.6 g) for 28 d. The LI and U-XOS-supplemented low-iron diet contained 0.4 mg Fe/100 g without any ferrous citrate in the mineral mixture. The U-XOS-supplemented LI diet contained up to 2% U-XOS by substituting U-XOS for cellulose. Blood was drawn from the tail vein of all of the animals every 4 d during the experimental period. At the end of each study period, the rats were euthanized by cervical dislocation under ether anesthesia during the early phase of the light cycle in a non-fasting state, 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.

In experiment 2, the rats were divided into two groups on the basis of body weight. Each group was fed a U-XOS-supplemented diet (X group, n=5, body weight: 228.1±2.3 g) or a control diet (C group, n=6, body weight: 229.8±3.0 g) for 7 d. The U-XOS-supplemented diet contained up to 2% U-XOS by substituting U-XOS for cellulose. Feces samples were collected for 3 d from IDA in rats in order to investigate the iron bioavailability properties of U-XOS in a previous study (19). As the result, we demonstrated that U-XOS promotes recovery from IDA by enhancing serum iron and the serum transfer saturation level during the early stage of the recovery process.

Table 1. Composition of experimental diets.

|                | Low-iron diet | U-XOS-low iron diet | Control diet | U-XOS diet |
|----------------|--------------|---------------------|--------------|------------|
| Casein         | 20.0         | 20.0                | 20.0         | 20.0       |
| α-Starch       | 45.7         | 45.7                | 45.7         | 45.7       |
| Sucrose        | 22.8         | 22.8                | 22.8         | 22.8       |
| Mixed oil      | 5.0          | 5.0                 | 5.0          | 5.0        |
| Vitamin mixture| 1.0          | 1.0                 | 1.0          | 1.0        |
| Mineral mixture| 3.5          | 3.5                 | 3.5          | 3.5        |
| Cellulose      | 2.0          | 2.0                 | 2.0          | 2.0        |
| U-XOS 4        | —            | —                   | 180.0        | 180.0      |

1 Rapeseed oil/soybean oil ratio=7/3.
2 AIN-76 vitamin mixture (per g mixture): vitamin A, 400 IU; vitamin D, 100 IU; vitamin E, 5 mg; vitamin K, 0.005 mg; vitamin B, 0.6 mg; vitamin B, 0.6 mg; vitamin B, 0.7 mg; vitamin B; 0.001 mg; vitamin B, 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.
3 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.
4 U-XOS (g/kg U-XOS): water content, 28; protein content, less than 1; lipid content, less than 1; iron content, undetectable; 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.
day 5 of the test period to evaluate absorption of iron.

**Blood constituent analysis.** The hemoglobin concentration and hematocrit level were measured using a hematology analyzer (KX-21NV; Sysmex Corp., Kobe, Japan). Serum iron and unsaturated iron binding capacity (UIBC) were measured using Detaminer Fe and UIBC (Kyowa Medix Co., Ltd., Tokyo, Japan) with an automatic biochemical analyzer (CL-8000; Shimadzu Corp., Kyoto, Japan). Total iron binding capacity (TIBC) and serum transferrin saturation were calculated as follows:

\[
TIBC = \frac{\text{serum iron} + \text{UIBC}}{\text{serum iron/TIBC} \times 100}
\]

**Estimation of gene expression.** Total RNA was isolated from the homogenized mucosa and liver samples using the Total RNA Isolation mini kit (Agilent Technologies, Inc., Santa Clara, CA), and converted to cDNA using a reverse transcriptase enzyme, ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan), according to the manufacturer’s instructions. Each target DNA fragment was amplified using the respective TaqMan gene expression assay kit and a real-time PCR system using cDNA as a template. The real-time polymerase chain reactions (PCR) for gene expression analysis were performed using DNA Engine Opticon and Opticon Monitor software (Bio-Rad Laboratories, Inc., Hercules, CA). TaqMan primer pairs/probes for the gene analysis were obtained using a TaqMan Gene Expression Assay (Applied Biosystems, Inc., Carlsbad, CA): Rn00565927_m1: DMT1, Rn00591187_m1: Ferroportin, Rn00667869_m1: β-actin. Reactions were performed with 10 μL of Premix EX Taq (Takara Bio, Inc., 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 s, 50 PCR cycles were performed as follows: 95˚C for 7 s, 60˚C for 30 s, and 72˚C for 20 s. The cycle thresholds of the genes of interest were compared with the housekeeping gene β-actin to determine relative changes in expression.

**Iron content of hepatic tissue.** Liver samples were perfused by saline, and treated by the wet ash method using a microwave extraction system (Ethos; Milestone Srl., Sorisole, Italy). The ash was suspended in dilute hydrochloric acid solution after evaporation, and left to dry. Iron concentrations were measured by polarizing Zeeman-effect atomic absorption spectrometry (Z-6100; Hitachi, Ltd., Tokyo, Japan) after suitable dilution. We determined that the coefficient of variation was 0.04. Iron concentrations were expressed on a wet-weight basis.

**Iron content of diets and feces samples.** We measured the weight of the collected feces and milled them to a size appropriate for analysis. The samples were then subjected to a series of chemical analyses to determine the iron content. The results were expressed as micromoles per gram of sample.

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**Table 2.** Body weight gain, food intake, blood parameters, and liver iron content on day 28 after the start of the study in experiment 1.

|       | LI  | LI-X | C   |
|-------|-----|------|-----|
| Body weight gain (g/d) | 0.4±0.1 | 0.2±0.1 | 0.5±0.1 |
| Food intake (g/d)      | 13.0±0.5 | 12.1±0.2 | 12.5±0.3 |
| Hematcrit level (%)    | 40.4±3.4 | 40.8±1.6 | 42.2±1.1 |
| Hemoglobin concentration (g/dL) | 13.4±0.8 | 13.3±0.5 | 14.0±0.5 |
| Liver iron content (μg/g liver, wet weight) | 42.9±4.3 ab 55.8±6.0b 78.0±11.8b |

Normal rats were fed a low-iron diet (LI: n=6), a U-XOS-supplemented low-iron diet (LI-X: n=7) or the control diet (C: n=6) for 28 d. The data were analyzed by 1-way analysis of variance (ANOVA), followed by the Tukey-Kramer test. Values with an unlike letter are significant: p<0.05.
fine powder after dehydration at 50°C for 3 d. Diets and the powdered feces were suspended in a dilute hydrochloric acid solution after being ashed at 550°C overnight by an electric furnace (FM37; Yamato Scientific Co., Ltd, Tokyo, Japan). Iron concentrations in the ash sample solutions were measured by the same method as that of serum iron. The iron absorption rate in this study was calculated as the apparent absorption rate as follows:

\[ \text{The apparent iron absorption rate (%)} = 100 \times \frac{\text{[total iron intake – iron excretion in feces]}}{\text{total iron intake}} \]

Statistical analysis. Data were presented as means± standard error (SE). 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 (Table 2, Figs. 2 and 3), or Student’s t test (Table 3). The level of significance was set at \( p<0.05 \).

RESULTS

Experiment 1

On day 28 after the start of the study, no significant differences in body weight gain, food intake, hemacrit level or hemoglobin concentration were observed among the three groups. The LI 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 C groups (Table 2).

The TBIC of the LI group statistically increased compared with that of the C group on day 21 and 24 after the start of this experiment. On day 28, the TBIC of the LI group was higher than that of the C group, but no significant difference was shown among the C, LI, and LI-X groups (Fig. 2A). The serum iron level of the LI-X group was increased compared with that of the C group, but no significant difference was shown among the three groups until day 6 from the start of the study. On day 28, the serum iron level of the C group was remarkably higher than that of the LI group (Fig. 2B). The LI-X group showed a remarkable significant increase in the transferrin saturation level compared with that of the LI group until day 6 from the start of this experiment. The increase of the transferrin saturation level of the LI-X group became less significant but remained higher than that of the LI group. On day 24, the transferrin saturation level of the LI-X group became remarkably higher than that of the LI group again, but no significant difference was demonstrated between the LI-X and C groups (Fig. 2C).

The DMT1 and ferroportin mRNA expression levels of the LI group were remarkably higher than that of the C group. In contrast, the increase in the mRNA expression of the LI-X was inhibited, compared with that of the LI group, and no significant difference was shown in the DMT1 or ferroportin mRNA expression levels between the LI-X and C groups (Fig. 2C).

Fig. 3. Iron transporter DMT1 (A) and ferroportin (B) mRNA expression in the first segment of small intestines (upper side, 1/4th) on day 28 after the start of the study in experiment 1. Normal rats were fed a low-iron diet (LI: \( n=6 \)), a U-XOS-supplemented low-iron diet (LI-X: \( n=7 \)) or the control diet (C: \( n=6 \)) for 28 d. Values are represented as means±SE. Values with an unlike letter are significant: \( p<0.05 \). The data were analyzed by 1-way analysis of variance (ANOVA), followed by the Tukey-Kramer test.

![Fig. 3](image-url)

Table 3. Body weight gain, food intake, and apparent iron absorption rate on day 7 after the start of the study in experiment 2.

|       | X     | C     |
|-------|-------|-------|
| Body weight gain (g/d) | 7.0±0.1 | 7.4±0.4 |
| Food intake (g/d) | 18.4±0.3 | 19.3±0.5 |
| The apparent iron absorption rate \(^1\) (%) | 64.1±2.1\(^a\) | 55.8±1.6\(^b\) |

\(^1\) The apparent iron absorption rate=100×[(total iron intake – iron excretion in feces)/total iron intake].

between the LI-X and C groups (Fig. 3A, 3B). A correlation between the level of the mRNA expression of the iron transporters and hepatic iron content were demonstrated in both groups.

Experiment 2

No significant differences in body weight gain or food intake was observed between the two groups, suggesting that the U-XOS did not affect the growth of rats. The apparent iron absorption rate of the X group significantly increased compared with that of the C group (Table 3).

DISCUSSION

In the present study, we investigated whether or not U-XOS was useful in the prevention of iron deficiency. Our results demonstrated that U-XOS prevented a low hepatic iron level in adult female rats fed a low-iron diet.

In experiment 1, at the end of the experiment period, no significant difference was shown in the hemacrit or hemoglobin levels between the groups. However, the LI-X and LI groups showed a significant decrease in serum iron, compared with the C group. Therefore, we diag-
nosed the LI-X and LI groups as iron deficient without anemia. In order to recruit iron from storage and get the iron into the serum when iron deficiency progresses in the body, the production of transferrin is enhanced in the liver (22). The TIBC of the LI-X and LI groups was higher than that of the C group, due to the iron deficiency. Additionally, the transferrin saturation level of the LI-X and LI groups decreased, compared with that of the C group, to correlate with the serum iron level. However, no significant difference was shown in the TIBC or transferrin saturation levels between the LI-X and LI groups. On the other hand, in the LI-X group, the decrease in the iron content in the liver was suppressed, compared with that of the LI group, suggesting that the progression of iron deficiency was delayed by the U-XOS. Because we performed frequent bloodletting on rats, not only was iron intake decreased but iron loss was also increased in the LI-X and LI groups. If the experiment period is shorter than this study or bloodletting is little, a significant difference might be demonstrated between the LI-X and LI groups.

The following three possibilities may be assumed in order to account for the findings in the present study that the hepatic iron content of the LI-X group was maintained compared with that of LI group, even though this group was fed a low-iron diet. The first possibility is an increase in dietary iron absorption by U-XOS. Because the food intake was at the same level, the amounts of ingested iron were also at the same levels in both the LI-X and LI groups. However, during the first 6 d, the serum iron and transferrin saturation levels of the LI-X group were increased, compared with the LI group. In experiment 2, we investigated the apparent iron absorption rate by rats fed a diet supplemented with U-XOS for 7 d. The apparent iron absorption rate was clearly increased by U-XOS. Therefore, an enhancement of serum iron at an early stage of the experimental period due to the U-XOS might have increased dietary iron absorption without mobilization of storage iron in experiment 1. Accordingly, we assumed that the recruitment of storage iron in rats fed a low-iron diet was delayed by the U-XOS. The iron of the low-iron diet in this study originated from the casein and α-starch. Most of iron contained in the casein is bound to phosphate groups, and lactoferrin or iron bound to citrate were also contained in crude components. Hence, it has been assumed that most of the dietary iron in this study is nonheme iron. Therefore, it appears that U-XOS may contribute to promoting the absorption of dietary nonheme iron.

The second possibility is inhibition of iron loss from the small intestine by U-XOS. We used adult rats in order to remove the effect of iron demand by growth in experiment 1. If food consumption is decreased by the ingestion of U-XOS, it has been assumed to reduce the iron excretion in the digestive tract or the cycle of detachment of small intestinal mucosal cells by inhibition of metabolic function. In this study, no significant difference was shown in body weight gain or food intake among the three groups, but those of the LI-X group showed a lower tendency compared with the LI and C group, and it is not possible to deny that U-XOS inhibited loss of iron.

The third possibility is an increase in iron bioavailability by U-XOS. Indigestible oligosaccharides such as U-XOS reach the large intestine without undergoing enzymatic degradation, are fermented by intestinal bacteria (23), and produce short chain fatty acid and organic acid (24). It has been reported that the short chain fatty acid produced by the fermentation promotes the cell proliferation of small and large intestinal epithelial cells (25). Accordingly, there is the possibility that U-XOS promotes iron absorption through the proliferation of small intestinal epithelium cells. Ninety-five percent or more of the short chain fatty acids produced by the bacteria in the large intestine is absorbed from the cecum and colon (26). Most of these short chain fatty acids are used as energy by the intestinal epithelium cells, and a smaller part are used for energy and steatogenesis in the liver through the hepatic portal vein (27). Therefore, the short chain fatty acids might be involved in iron metabolism in the liver. It is well known that serum lactic acid is converted to glucose in the liver or kidney. In the present study, there is the possibility that the lactic acid transferred to the liver may be involved not only in the lactic acid cycle, but also in iron metabolism. One of the limitations of this study is that we did not assay the short chain fatty acids or the organic acid levels. Further studies should be conducted to investigate the correlation between iron metabolism, the short chain fatty acids and the organic acid in the liver. Furthermore, we should also examine the efficacy of U-XOS in regard to iron metabolism, such as the iron balance under a low-iron diet, and the mechanism of promotion of dietary nonheme iron absorption by U-XOS.

In conclusion, we demonstrated that U-XOS preserved the storage of hepatic iron in adult female rats fed a low-iron diet, suggesting that dietary supplementation with U-XOS could prevent IDA and that it might be beneficial in the amelioration of the biological utilization of iron.

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