Effect of Transferrin on Enhancing Bioavailability of Iron

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Transferrin was isolated and purified from bovine plasma. An intestinal segment in situ experiment showed that 19.2% of injected iron was absorbed when FeCl₃ (80 µg Fe/ml) was injected into a duodenum segment of iron-deficient rats. With addition of 10 and 20 mg of purified transferrin/ml, however, ratios of absorbed iron through duodenum segments were significantly increased to 52.7 and 57.9%, respectively. After transferrin-rich extract was isolated by batch type ion exchange chromatography, a soluble ferric complex of the transferrin extract was prepared by adding ferric salts to transferrin extract followed by dialysis, sterilization, and freeze drying. Results of the animal experiment for comparing bioavailabilities of different irons showed that irons in Fe-transferrin extract was most efficiently absorbed and incorporated into hemoglobin generation in anemic rats.

Key words: iron; transferrin; bioavailability; plasma

Iron deficiency anemia is the most prevalent nutritional disorder in the world. Especially, infants under 2 years of age, teenage girls, pregnant women, and the elderly are at high risk of iron deficiency anemia. Iron fortification with micronutrients is one of the least expensive and more effective ways of supplying iron to target people. However, the biological availability of added iron has shown to be extremely variable. For increasing the biological availability of iron, iron should be in a soluble form in the duodenum where most irons are absorbed. Many factors, such as chemical forms of iron and diet composition, are known to influence the biological availability of iron.₁⁻³ Iron absorption can be inhibited to a varying degree by a number of ingredients, including carbonates, oxalates, phosphates, and phytic acid.₄,⁵ Otherwise, ascorbic acids and certain proteins in foods are known to increase iron absorption.⁶,⁷

Transferrin, which composes about 7–10% of plasma proteins, is a non-heme iron binding glycoprotein. Because of its strong iron binding ability, transferrin plays a role in transferring iron from storage areas to erythroblasts.⁸ Therefore, transferrin can be developed as a functional food protein that binds irons to maintain irons in a soluble monomeric form at neutral pH of a duodenum. Main objectives of this study were to isolate transferrin from bovine plasma and investigate its effects on increasing iron’s bioavailability through in vitro and in situ experiments.

Materials and Methods

Plasma. Bovine blood was obtained from the municipal slaughterhouse. For preventing blood coagulation, sodium citrate was immediately added to blood with a final concentration of 1.0% of blood. Collected blood was centrifuged at 5,300 × g for 30 min and supernatant (plasma) was collected and stored at −40°C.

Purification of Transferrin. Plasma was fractionated at room temperature by a batch-type ion exchange technique using Q Sepharose Fast Flow matrix (Pharmacia Biotech AB, Uppsala, Sweden). The matrix was washed twice with distilled water, and the binding buffer (10 mmol Na₂HPO₄, pH 10.0) was added in an amount equivalent to two times the volume of the gel and stirred for 2 h to equilibrate. Then, plasma was added in an amount equivalent to half of the gel volume and stirred for another 20 min. The fraction of unbound proteins, which was mainly composed of transferrin and immunoglobulins, was collected through a glass filter by a hand-operated vacuum pump, concentrated and lyophilized. A transferrin-rich fraction was further purified by a reverse-phase column on HPLC (Jasco Co., Model LC-900, Tokyo, Japan) fitted with Crest pack C₁₈ column (Jasco Co., Tokyo, Japan) and UV detector. Mobile phases used for separation were 0.1% trifluoroacetic acid/H₂O (A) and 0.1% trifluoroacetic acid/acetonitrile (B). A transferrin-rich fraction was eluted by changing the gradient of eluting buffers from 70/30 (A/B) to 50/50.

Electrophoresis. Protein composition of each fraction from batch-type ion exchange chromatography and HPLC were analyzed by SDS-PAGE.⁹ For this experiment, 9% acrylamide separation gel was used for protein separation and PhastGel Blue R for staining reagent. Protein composition of the fraction was estimated by a laser densitometer (Pharmacia Ultra Scan XL, Uppsala, Sweden).

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Amino acids composition. Amino acid compositions of purified transferrin and standard bovine transferrin (Sigma Chemical Co., St. Louis, Mo., U.S.A) were compared by a Pico-Tag system (Waters PicoTag system, Palo Alto, Ca, USA).

Solubility of iron at duodenum conditions. The effect of purified transferrin on iron’s solubility at duodenum conditions was measured by incubating FeCl₃ (7.5 μg Fe/ml) with or without transferrin at pH 6.0, 37°C for 2h. After incubation, iron solutions were centrifuged at 5,000 × g for 30 min and concentrations of soluble iron in a supernatant were measured by the Ferrozine assay.¹⁰)

Effects of transferrin on bioavailability of iron (in situ). The effects of transferrin on effective absorption of iron into a body was examined by ligated intestinal segment in situ technique. Male, weanling SD rats were housed in mesh-bottom plastic cages in a controlled environment. Iron-deficient diet (Table 1) and deionized water were offered ad libitum for 8 weeks to induce iron-deficiency anemia. These rats were then divided into three groups of 10 rats. They were anaesthetized intraperitoneally with sodium pentobarbital. Through a medical laparotomy, the pancreatic-biliary duct was ligated at its drainage into the duodenum. The inside of the duodenum segment was cannulated at its entry and exit, then ligated at its drainage into the duodenum. The duodenum was cannulated at its entry and exit, then washed with isotonic solution. Table 2 shows the compositions of the solution injected to the control and transferrin added (10 and 20 mg, respectively) groups. Radio-active ⁵⁹FeCl₃ (0.5 μCi/ml) was spiked into 80 mg of Fe/ml in 0.01 M HCl. One ml of the solution for each group was injected into the upper portion of the duodenum through a syringe and the abdomen was closed using a Michel clip. The animals were then maintained in a quiet room at 23°C for 1 h. Then, the abdomen was disclosed and both sides of cannulated ends of duodenum segment were cut and separated from the intestine. The inside of the duodenum segment was thoroughly washed with deionized water and washing solution and duodenum segments were collected for determination of ⁵⁹Fe activities. Concentrations of ⁵⁹Fe in washing solution and duodenum segment were measured by a gamma counter (Packard Auto-Gamma Model 2000 series, Meriden, CT, USA). All ⁵⁹Fe counting data were corrected for decay and counting efficiency.

Fe-Transferrin Extract Complex. For the animal feeding test, 100 ml of transferrin-rich fraction obtained from batch-type ion exchange chromatography was mixed with 20 ml of FeCl₃ solution containing 1 g of Fe³⁺. The mixture was poured into a dialysis bag (MWCO 6,000~8,000) and dialyzed against an excessive deionized water at 10°C for 24 h to remove free iron. After dialysis, the mixture was sterilized at 121°C for 15 min to give microbial safety to the product and lyophilized for further experiments. Subsequently, the concentration of iron in Fe-transferrin extract complex was analyzed by both ferrozine assay and an I.C.P (Jobin Yvon, JY138 ultracore, France).

Animal Experiment. For finding suitable amounts of iron for the daily dosage, the marginal dosage experiment was done previous to the main experiment. Three-week-old male weanling SD rats were housed in mesh-bottom plastic cages under a controlled environment. The iron-deficient diet and deionized water were offered ad libitum until their hemoglobin (Hb) level dropped to 7 g/dl or below. Then, anemic rats were randomly divided into three groups of 10 rats and continuously fed an iron-deficient diet. Except the control group, all rats were fed FeSO₄ once a day in the morning at three different levels of 25, 50, and 100 μg Fe dissolved in 1 ml of deionized water. Weight gains of all rats were recorded weekly. Hemoglobin concentrations and hematocrit (%) were measured from blood samples that were collected into heparinized vessels from the orbital vein plexus at 7 days intervals.

Table 2. Formulation of Iron Complexes Injected into in Situ Ligated Segment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control (ml)</th>
<th>Transferrin-10 mg (ml)</th>
<th>Transferrin-20 mg (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeCl₃¹)</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Transferrin</td>
<td>—</td>
<td>10 mg</td>
<td>20 mg</td>
</tr>
<tr>
<td>0.01 M HCl</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Deionized water</td>
<td>0.542</td>
<td>0.542</td>
<td>0.542</td>
</tr>
<tr>
<td>0.25 M/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tris buffer</td>
<td>0.058</td>
<td>0.058</td>
<td>0.058</td>
</tr>
</tbody>
</table>

Final pH: 7.2
¹) Mixture of 80 mg Fe/ml in 0.01 M HCl and ⁵⁹FeCl₃ (0.5 μCi/ml).

Table 1. Composition of Iron-deficient Diet

<table>
<thead>
<tr>
<th>Components</th>
<th>Iron deficient diet (g/kg dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200</td>
</tr>
<tr>
<td>Corn starch</td>
<td>150</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>3</td>
</tr>
<tr>
<td>Mineral mixture⁶</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mixture⁶</td>
<td>10</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>500</td>
</tr>
<tr>
<td>Corn oil</td>
<td>50</td>
</tr>
</tbody>
</table>

¹) The mineral supplement of diet contained (g/kg): calcium phosphate 500.0, sodium chloride 74.0, potassium citrate monohydrate 220.0, potassium sulfate 52.0, magnesium oxide 24.0, manganese carbonate monohydrate 3.5, zinc carbonate monohydrate 1.6, copper carbonate anhydrous 0.3, potassium peroxide 0.01, sodium selenite monohydrate 0.01, chromium potassium sulfate 0.55, and finely ground sucrose 124.03.

⁶) The vitamin supplement contained (g/kg): thiamin HCl 0.6, riboflavin 0.6, pyridoxine HCl 0.7, niacin 3.0, calcium pantothenate 1.6, folic acid 0.2, biotin 0.02, vitamin B₁₂ 1.0, dry vitamin A palmitate 0.8, dry vitamin E acetate 10.0, vitamin D₃ 0.25, menadione sodium bisulfite complex 0.15, and sucrose fine powder 981.08.
For the main animal experiment of investigating bioavailability of iron in Fe-transferrin extract complex, iron-deficient anemia was induced in rats as previously done in the marginal dosage experiment. Anemic rats were then divided into five groups of 10 rats each and continuously fed an iron-deficient diet. One group was orally administered the Fe-transferrin extract with equivalent amount of 50 µg Fe in 1 ml deionized water once a day. Other two groups were fed either FeSO₄ or FeCl₃ at 50 µg Fe level. To evaluate the effect of conjugation between iron and transferrin extract on increasing bioavailability of iron, the final group was fed FeCl₃ (50 µg iron) and transferrin extract (1.35 mg), separately. During the experimental period, body weight gains, hemoglobin concentrations, and hematocrit were analyzed by exactly the same procedure with those of the marginal dosage experiment. Since various weight gains in different groups should be considered for an exact evaluation of the amount of irons used in hemoglobin formation, the Hb value was converted to total Hb concentration according to the following formula:

\[
\text{Total Hb (g)} = \frac{\text{Hb (g/dl)} \times \text{body weight (g)}}{0.067/100}
\]

All animal experiments were performed under the guidelines of the Laboratory Animal Experiment Committee of Korea Food Research Institute.

**Results and Discussion**

*Purification of transferrin*

Bovine plasma was fractionated by a batch-type ion exchange technique. When the ion-exchange matrix was washed with the binding buffer (pH 10), 31% of total plasma proteins were eluted (Fig. 1). This fraction was mostly composed of IgG (47%) and transferrin (30.4%). Most of transferrin (90.4%) in plasma exhibited low affinity to the ion exchange chromatography (IEC) gel and was easily fractionated from other plasma proteins by the binding buffer. This transferrin-rich fraction was further fractionated on HPLC for transferrin purification. As shown in Fig. 2, transferrin was the first main peak eluted from the reverse phase column. Transferrin peaks were collected, concentrated by ultra-filtration, and freeze-dried. Purified transferrin showed identical amino acid compositions (Table 3) and mobility on SDS-PAGE with standard transferrin indicating that transferrin was successfully purified from bovine plasma by batch-type IEC and HPLC consecutively.

**Effects of transferrin on bioavailability iron**

Iron is known to be absorbed from the upper intestine (duodenum segment). Being soluble in the duodenum may be a prerequisite to iron absorption into a body. Iron
forms macromolecules with hydroxyl groups in an aqueous solution and ultimately precipitates as the pH becomes more alkaline. It has been estimated that some chelating agents can prevent the precipitation of iron in the nearly neutral environment of the intestine, thus rendering otherwise insoluble iron available for absorption.\textsuperscript{11}) To study the effect of transferrin on solubility of ferric iron in duodenum conditions, ferric iron (7.5 \( \mu \)g/ml) was incubated at pH 6, 37°C for 2h. After incubation, only 2.2% of added iron were solubilized in a supernatant (Fig. 3). However, the percentage of soluble iron in a supernatant was rapidly increased to 47.0% when 1.0 mg/ml of transferrin was added and incubated together. With addition of 2.5 and 5.0 mg transferrin/ml, the percentage of iron in supernatants increased up to 76.9 and 96.0%, respectively. Although the data on serum total iron, unsaturated iron binding capacity (UIBC), and total iron binding capacity (TIBC) of the purified transferrin revealed that 18% of the purified transferrin was saturated with irons (not shown), addition of transferrin can increase solubility of iron at duodenum condition.

The next step was done to investigate the effect of transferrin addition on biological availability of iron. The absorption of iron by a body is complex and affected by many factors. It is estimated that less than 10% of iron in food is absorbed by adults with normal Hb levels, although absorption can be increased with an iron deficiency.\textsuperscript{12,13}) In this experiment, test rats were fed an iron-deficient diet to induce iron-deficiency anemia so as to have nearly equal iron-absorption conditions. Since the normal hemoglobin level is considered to be around 15 g/dl, all test rats which showed hemoglobin level ranging from 5.5 to 7.4 g/dl were considered to be in the state of iron-deficient anemia. A ligated intestinal segment in situ study, there is good evidence that transferrin increases biological availability of iron.

\textit{Processing Fe-transferrin extract complex}

Since the transferrin-rich fraction was easily and massively isolated from wasted bovine plasma by the batch-type ion exchange technique, the next step was to find practical and economical ways of using this fraction in increasing the bioavailability of iron. The best answer was to develop an iron-transferrin extract complex of more or less tightly bound irons to transferrin extract. This complex was prepared by mixing a transferrin-rich fraction (fraction B) from batch-type ion exchange technique and \( \text{FeCl}_3 \) solutions followed by dialysis, sterilization and lyophilization. According to Ferrozine and an atomic absorption spectrophotometer results, average of 37% of added irons were bound to transferrin extract representing 37 mg Fe/g Fe-transferrin extract. Freeze-dried Fe-transferrin extract was readily dissolved in water. Although some major plasma proteins such as albumin and immunoglobulin were treated with iron in
the same manner as iron-transferrin extract complex, they failed to form complexes with irons.

**Bioavailability of Fe-transferrin extract complex**

From the marginal iron dosage result, rats that were daily administered 50 μg Fe showed no significant changes in Hb values, 5.7 at day 0 to 5.99 g/dl at day 21 (Fig. 5). When rats were fed 25 μg Fe, however, Hb levels were slightly decreased from 5.7 at day 0 to 5.04 g/dl at day 21, while Hb levels were increased from 5.7 at day 0 to 6.96 g/dl at day 21 in the 100-μg-Fe fed group. These results indicated that bioavailability of irons in anemic rats would be evaluated more precisely by feeding 50 μg Fe per day.

For the main animal experiment, rats were induced to iron deficient anemia by taking an iron deficient diet until Hb levels were dropped to lower than 7 g/dl. Then, the equivalent amount of 50 μg Fe of Fe-transferrin extract, FeSO₄, and FeCl₃ with or without transferrin extract, were fed daily. The control group was continuously fed an iron-deficient diet without oral administration of any iron. Weight gains of iron administered rats were significantly higher than those of the control group for 42 days (Fig. 6). This assured the fact that iron is essential for a proper growth of animals. Although statistically not significant, the Fe-transferrin extract fed group seemed to gain weights better than other iron-administered groups indirectly indicating that iron bound to transferrin extract was more readily utilized for growth.

Figure 7 showed changes in total Hb concentrations of iron-administered rats. Although total Hb concentration of the control group was constant around 0.8 g for 42 days, that of the Fe-transferrin group was increased from 0.783 g at the beginning to 2.29 g after 42 days. Total Hb in FeSO₄ and FeCl₃ fed groups were rather slowly increased from 0.760 and 0.727 to 1.118 and 1.101 after 42 days, respectively. It is widely accepted that ferrous iron (Fe²⁺) is more soluble than ferric iron (Fe³⁺) resulting in more rapid uptake into a mucosal cell. Our study showed a similar result that ferrous irons in FeSO₄ were more readily absorbed and used in hemoglobin generation than ferric irons in FeCl₃.

When FeCl₃ and transferrin extract were separately fed to anemic rats, total hemoglobin concentrations were more rapidly, but statistically insignificant, increased than those of FeCl₃ solely fed group. This result suggested that addition of transferrin extract could enhance absorption of irons. However, as previously shown, the effects of transferrin extract on bioavailability of iron were much more efficient when it formed a complex with iron.

Changes in hematocrit in five groups are shown in Fig. 8. Except Fe-transferrin extract group, which had distinctively increased hematocrit values from 16.8 to 22.9 after 42 days, other iron-administered groups didn’t show any statistically significant changes in hematocrit during the experimental period.

Plasma is a potential source of various proteins. It has been reported that plasma proteins have good rheology-related functional properties such as gelation and
emulsification, so their usage in food processing has a lot of advantages.\(^{17,18}\) Like a lactoferrin, that is an iron-binding glycoprotein found in externally secreted fluids such as milk to enhance neonate’s iron absorption,\(^{19,20}\) transferrin is also an iron-carrying glycoprotein in blood. Since both lactoferrin and transferrin have similar molecular weight, amino acid composition, and iron-binding properties, more efforts should be invested to develop ways to use transferrin as a functional food ingredient.

**Conclusion**

Bovine transferrin was purified by batch-type ion exchange chromatography and HPLC. Purified transferrin increased solubility of irons and their absorption into a body. When irons formed complex with transferrin extract, they were effectively incorporated into hemoglobin generation. Although the mechanism involved with such effective absorption of iron in Fe-transferrin extract remains to be elucidated, transferrin extract seemed to play an important role in enhancing solubility of irons at duodenum.

**References**