Proline-Rich Proteins Moderate the Inhibitory Effect of Tea on Iron Absorption in Rats

Hee-Seon Kim1 and Dennis D. Miller*

Department of Food Science and Nutrition, Soonchunhyang University, Asan, Chungnam, 336–745, Korea and *Department of Food Science, Cornell University, Ithaca, NY 14853

ABSTRACT Tea inhibits iron absorption in studies in which tea is given with radiolabeled iron to humans as a single dose. Our objective was to test the hypothesis that proline-rich proteins (PRPs) may act as a defense against this effect by forming complexes with tannins, thereby preventing them from inhibiting iron absorption. Two studies were conducted. In study 1, rats were given test solutions containing 59FeCl3 in water, tea, or tea + gelatin (T/G). In study 2, the rats were divided into 3 groups and assigned to one of 3 nutritionally complete diets: control, tea (5 g tea tannin/kg diet), or T/G (5 g tea tannin + 60 g gelatin/kg diet). Rats were fed the respective diets for 5 d and then given a single 59Fe-labeled meal of the diet. Iron absorption was measured by whole-body retention of the 59Fe over a 2-wk period. Iron absorption in study 1 was lower in the tea group (24 ± 9.6%, P < 0.05) than in the T/G (42 ± 19.4%) or water groups (50 ± 7.5%). In study 2, iron absorption did not differ among the groups. Rats fed the tea diet had dramatic hypertrophy of the parotid salivary glands. Adding gelatin as a proxy for salivary PRPs to the tea eliminated the inhibitory effect of tea on iron absorption. The results suggest that PRPs, whether from salivary glands or diet, can protect against the inhibition of iron absorption by tea.

KEY WORDS: • iron absorption • tea • phenolic compounds • proline-rich proteins • rats

Tannins in foods are associated with toxic and antinutritional effects including reduced food intake, growth retardation, and impaired nutrient absorption (1). In contrast, recent studies demonstrated potent antioxidant activity of tea polyphenolics, leading to suggestions that tea drinking may reduce the risk for heart disease and cancer (2). Drinking tea with meals reduced nonheme iron absorption in rats (3,4) and humans (5–7). Disler et al. (8) attributed the inhibitory effect of tea on iron absorption to the insoluble iron-tannin complex that forms in the lumen of the gastrointestinal tract. However, this putative role for tannins is not fully consistent with existing evidence. In cases of single-dose studies such as stomach tube and ligated loop administration in animals and ingestion of a single radiolabeled meal in both animal (3,9–11) and human studies (7,8,11–12), iron absorption was reduced with tea. However, when the experimental period was extended, results were variable. In rat studies, when the experimental period was longer than 7 d, tea ingestion did not inhibit iron absorption as measured by iron retention (9,13,14). In contrast, tea ingestion for 28 d reduced liver iron in rats (4). Epidemiologic evidence is also conflicting. One study based on data from the second National Health and Nutrition Examination Survey reported a negative correlation for tannins. Most of the condensed tannin-PRP complexes

1 To whom correspondence should be addressed. E-mail: hskim1@sch.ac.kr.

2 Abbreviations used: PRP, proline rich protein; T/G, tea + gelatin diet; TCA, trichloroacetic acid.
also remained insoluble under conditions similar to those in the stomach and small intestine, supporting the hypothesis that PRPs act as a defense against tannins (19).

Conflicting results from epidemiologic and clinical studies and short-term feeding trials may be due to the duration of experiments in which the adaptation to tannins may or may not have begun. Therefore, we hypothesized that tea inhibits iron absorption in unadapted animals but that chronic exposure will stimulate the production of PRP, which will counteract the inhibition. Because little is known about the effects of PRPs on iron-tannin complex formation and stability, we compared iron absorption in rats given a single dose of a tea-iron mixture with rats given tea over an extended period of time. Gelatin was chosen as a proxy for salivary PRPs because of its high proline content, high affinity for tannins (23), and ready availability.

**MATERIALS AND METHODS**

Study protocols were approved by the Institutional Animal Care and Use Committee at Cornell University.

**Expt. 1: effect of a single exposure to tea on iron absorption**

**Preparation of tea.** Black tea (10 g; U.S. Tea Association, Black Tea Research Blend, Thomas J. Lipton) was added to 1 L boiling distilled, deionized water and left at room temperature for 5 min. The brewed tea was filtered (Whatman no. 1 qualitative, Whatman), and tannin concentration was determined by a method for determining the concentration of iron-binding phenolic groups (25). The brewed tea contained 118 ± 1.1 mg/L catechin equivalents (mean ± SD of triplicates) and 82 ± 2.0 mg/L tannic acid equivalents.

**Animal care.** Male Sprague-Dawley rats (Camr Research Institute) weighing ~300 g (range 280–320 g) were caged individually in suspended stainless steel cages with wire-mesh bottoms in a temperature controlled room (20°C) with a reverse 12-h light:dark cycle. The rats had free access to a commercial diet (Prolab 1000, Agway) and distilled water for 3 d before the study to allow them to adjust to a new environment. Blood samples were obtained from the tail and hemoglobin concentrations were determined by a cyanmethemoglobin method (Sigma kit 525-A, Sigma Chemical).

**Iron absorption measurements.** Test solutions with 0.18 mmol Fe/L were prepared. These solutions contained water, tea, or tea with gelatin (Expt. 1; A) and in rats given a single meal extrinsically labeled with 59Fe after consuming control, tea, and tea + gelatin diets for 5 d (Expt. 2: B). Counts at zero time are set at 100% and subsequent counts are expressed as a percentage of the initial count. Values are presented as the natural logs. The percentage absorptions were calculated by extrapolating the linear portion of the curves (72–288 h) to zero time and converting the intercept to the antilog. Values shown are for a single representative animal in each group.

**Expt. 2: effect of chronic exposure to tea on iron absorption**

**Diet formulation.** Test diets were formulated to meet recommended nutrient levels for rats (AIN-76A) (27,28). The 3 diets, standard AIN-76A, tea, and T/G, contained 20% protein provided as either casein (95% protein, dry basis, ICN Biochemicals) or gelatin (Flake-50 bloom, food-grade type A, ICN Biomedicals). Tryptophan, phenylalanine and tyrosine, limiting amino acids in gelatin, were added to make the amino acid score of gelatin-containing diets similar to that of the other diets. Compositions of the test diets are shown in **Table 1**.

Tea was brewed as described previously (29). Briefly, 40 g black tea was added to 1 L boiling distilled deionized water and allowed to steep at room temperature for 30 min. The brewed tea was filtered (Whatman no.1 qualitative, Whatman). Tannin concentration was 211 ± 2.5 mg/L as catechin equivalents (mean ± SD of triplicates) and 369 ± 2.5 mg/L as tannic acid equivalents, determined by measuring the concentration of iron-binding phenolic groups (25). Then, 5 L of brewed tea was mixed with 2 kg of the dry diet and the mixture was freeze-dried and ground (Sample mill, Cemotoc, 1090, Tecator). This diet contained 5.0 g tannin as tannic acid equivalents/kg diet (Table 1). The T/G diet was prepared by mixing 5 L of tea and 68 g of gelatin, stirring, and leaving to stand for 15 min. The T/G mixture was then mixed with 1.932 kg of the dry diet without gelatin. The diet mixture was freeze-dried and ground with a bench top mill (Sample mill, Cemotoc, 1090, Tectator). This diet yielded 5.0 g tannic acid equivalents/kg diet. Results of diet analyses for protein content (30), tannin concentration (25), and total iron concentration (31) are shown in Table 1. The diets were stored at 4°C until used.

**Animal care.** Male Sprague-Dawley rats (Camr Research Institute) weighing ~300 g (range 260–350 g) were housed individually and allowed 3 d to acclimate to the new environment as described in Expt. 1. The rats were then allocated to 3 groups of 6 male rats each with similar mean hemoglobin concentration and body weight. Groups of rats were assigned randomly to either the standard diet (control), the tea diet (Tea), or the T/G diet. Each group of rats was fed the appropriate test diet for 5 d (from d 1 to 5) before dosing with an 59Fe-labeled test meal. Body weights and feed intakes were monitored until 59Fe-labeled test meals were given.

**Assessment of iron absorption.** After the experimental diets were fed to the rats for 5 d, the rats were deprived of food for 14 h. Then the rats were fed a 2-g test meal of their respective diets labeled extrinsically with 59FeCl3 (18.5 kBq, specific activity of 1127 kBq/μg total Fe; Dupont/New England Nuclear). The test meals were offered...
TABLE 1
Composition of diets fed in Expt. 2

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Control</th>
<th>Tea</th>
<th>T/G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>200</td>
<td>200</td>
<td>166</td>
</tr>
<tr>
<td>Gelatin</td>
<td>—</td>
<td>—</td>
<td>343</td>
</tr>
<tr>
<td>Fat</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Corn oil</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>150</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Sucrose</td>
<td>500</td>
<td>490</td>
<td>487</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>35</td>
<td>33</td>
</tr>
<tr>
<td>Choline</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>AIN-76A vitamin mix</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>AIN-76A mineral mix</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Amino acid</td>
<td>—</td>
<td>—</td>
<td>0.12</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>—</td>
<td>—</td>
<td>1.82</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>—</td>
<td>—</td>
<td>0.06</td>
</tr>
<tr>
<td>DL-Tryptophane</td>
<td>—</td>
<td>—</td>
<td>0.06</td>
</tr>
<tr>
<td>Tea solids</td>
<td>—</td>
<td>—</td>
<td>25</td>
</tr>
<tr>
<td>Protein6</td>
<td>200.0 ± 0.5</td>
<td>189.4 ± 1.9</td>
<td>193.3 ± 2.4</td>
</tr>
<tr>
<td>Iron,7 mg/kg diet</td>
<td>35.0 ± 1.1</td>
<td>34.3 ± 2.3</td>
<td>34.6 ± 3.2</td>
</tr>
<tr>
<td>Tannic acid equivalents8</td>
<td>0.0 ± 0.0</td>
<td>5.0 ± 0.0</td>
<td>5.0 ± 0.8</td>
</tr>
</tbody>
</table>

1 Casein, 95% protein, dry basis.
2 Gelatin, Flaked-50 Bloom, Type A (ICN Biomedicals).
3 Mixed and added with tea solution.
4 AIN-76A diet (28).
5 Added as liquid tea.
6 Analyzed as Horwitz (30), values are means ± SD, n = 3.
7 Analyzed as Kapsokkeleu and Miller (31), values are means ± SD, n = 5.
8 Analyzed as Brune et al. (25), values are means ± SD, n = 3.

TABLE 2
Iron absorption calculated from whole-body retention of 59Fe in rats fed control diet and control diet containing tea.

<table>
<thead>
<tr>
<th>% Iron absorption</th>
<th>Control</th>
<th>Tea</th>
<th>T/G</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>51</td>
<td>24</td>
<td>42</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Protein content of the glands was estimated by measuring the absorbance of the extract at 230 nm. Briefly, thawed parotid glands were placed in 5 volumes (wt/vol) of ice-cold 0.61 mol/L Tris-HCl buffer, pH 7.4, containing 0.14 mol/L NaCl, and homogenized in an Omni-mixer (Falkter, Fisher Scientific) at top speed for 60 s. After centrifugation at 17,000 × g for 20 min (Sorvall RC-5B, Du Pont Instruments), TCA was removed from the supernatant by extraction with water-saturated ether 4 times, 4 volumes each time. The aqueous phase, designated the TCA-soluble fraction, containing the PRPs was collected.

Biochemical analyses. Parotid glands were rapidly excised, washed in saline solution, dissected free from adhering tissue, weighed, and stored at −20°C. Frozen parotid glands from rats in each group were thawed and the PRPs were isolated using a trichloroacetic acid (TCA) extraction procedure as described by Mehansho et al. (20). PRP content of the glands was estimated by measuring the absorbance of the extract at 230 nm.

Iron absorption calculated from the whole-body retention of 59Fe in rats was determined using a whole-body gamma scintillation spectrometer (Fig. 2). The rats had free access to the appropriate unlabeled diet throughout the experiment (Table 2). The feed efficiency of the tea group was negative initially, but eventually increased to that of the control group (Table 2).

RESULTS

Tea reduced iron absorption by >50% (P < 0.003) when given alone as a single dose by stomach tube in Expt. 1 (control, 50 ± 7.5% vs. tea, 24 ± 6.9%) but only by 17% (P = 0.282) when given with gelatin (iron absorption rate, 42 ± 19.4%, Fig. 2A). In contrast, when rats were fed the tea-containing diet for 5 d before they received the radio iron doses (Expt. 2), tea did not affect iron absorption (P = 0.855, Fig. 2B). Iron absorption was also not affected when gelatin was added to tea and incorporated into the diet. Final hemoglobin concentrations of the rats in Expt. 2 did not differ among the groups.

In Expt. 2, rats in the tea group lost body weight and consumed much less food than the control group (P < 0.01, Table 2) during the first 5 d. They started to regain body weight beginning on d 7 (data not shown). In rats fed the T/G diet, body weights did not differ from the controls. Feed intakes did not differ between control and T/G groups throughout the experiment (Table 2). The feed efficiency of the tea group was negative initially, but eventually increased to that of the control group (Table 2).

Significant (P < 0.01) hypertrophy of parotid glands occurred in the tea group during Expt. 2 (Table 3). The total amount of PRPs in parotid glands did not differ among the groups (P = 0.337). However, because the final weights of the rats in the tea group were lower (P = 0.003), the PRP level per kilogram body weight was higher in the tea group than in the control group (P = 0.012). The T/G group did not have heavier parotid glands (P = 0.997), and the production of PRPs did not differ from the control group (P = 0.339, Table 3). When the PRP levels were expressed relative to the weight of the parotid gland, the groups did not differ (P = 0.333). SDS-PAGE of proteins extracted with Tris buffer from parotid glands of the rats fed the tea diet for 5 d showed 2 more bands around 40–50 kDa compared with the control group (Fig. 3A). These proteins were confirmed as acid-soluble proteins.
line-rich salivary proteins using chromatography of TCA-soluble proteins of parotid gland extracts (data not shown) and gel electrophoresis (Fig. 3B).

**DISCUSSION**

Iron deficiency is a major world health problem that is caused, to a great extent, by poor iron absorption. Iron absorption from meals can be enhanced or inhibited by various food components. Tea, a widely consumed beverage, contains appreciable amounts of phenolic compounds. These compounds possess antioxidant activity and a putative beneficial role in the prevention of chronic diseases (2,14). However, studies by other investigators in both rats (3,4,9–11) and humans (5–8,11,12) showed that tea and phenolics related to those found in tea can have deleterious effects on growth, food consumption, and food utilization as well as on hematological variables. In contrast, a substantial body of evidence suggests that tea ingestion over prolonged periods does not significantly reduce iron absorption either in rats (9,13) or in humans (17). In the studies reported here, we wanted to show that the conflicting results of tea effects on iron absorption are due to differences in the duration of exposure to tannins because prolonged tannin ingestion may trigger defensive mechanisms against tannin toxicity. Our results provide evidence that rats exposed to tea for 5 d have heavier parotid glands and therefore, presumably, secrete more PRP in saliva. Rats in the tea group showed dramatic hypertrophy of the parotid glands, suggesting the initiation of a defensive reaction to tea ingestion. Because rats in the tea group weighed significantly less, the tea group secreted more PRP per gram body weight. This 5-d exposure may seem a short time for adaptation to occur; nevertheless, it reduced or eliminated the effects of tea on iron absorption. We selected a 5-d period of exposure because induction of rodent proline-rich translation products as well as the secretion of the proteins were reported to occur after only 3 d of tannin ingestion (20–22). It is noteworthy that when the 59Fe dose was administered by gavage, thereby by-passing the mouth and exposure to salivary proteins, tea did inhibit iron absorption (Fig. 2A). Zhang et al. (9) reported similar results. On the other hand, when rats were given tea either as a sole liquid source (9) or as a dried extract incorporated into the diet (the present study) and fed for several days, it did not inhibit iron absorption [(9), Fig. 2B].

Humans are expected to have a similar defensive response because PRPs are major components of saliva in humans as well as other animals (18,32). Lu and Bennick (19) reported that the protein in human parotid secretions is >70% PRP, and most of the condensed tannin-PRP complexes remained insoluble under conditions similar to those in the stomach and small intestine, supporting the hypothesis that PRP protects against the effects of tannins. More than 22 PRPs have been described in human saliva (33), and it was found in a survey of human saliva that 2 families of proteins, histatins and PRPs, were most effective in precipitating tannins (34). The hypothesis that large amounts of PRPs in the saliva of humans provide a defense against deleterious effects of dietary tannins is plausible because an epidemiologic study (15) showed a negative correlation between tea drinking and anemia. However, fur-

**TABLE 2**

<table>
<thead>
<tr>
<th>Body weight, g</th>
<th>Feed intake, g/d</th>
<th>Feed efficiency, g gain/g feed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d 0 (Initial)</td>
<td>d 1</td>
</tr>
<tr>
<td>Control</td>
<td>300.2 ± 20.6</td>
<td>302.3 ± 20.5</td>
</tr>
<tr>
<td>Tea</td>
<td>300.7 ± 34.1</td>
<td>297.6 ± 32.4</td>
</tr>
<tr>
<td>T/G</td>
<td>300.0 ± 20.7</td>
<td>302.6 ± 29.6</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n = 6.
2 Means in a column with superscripts without a common letter differ, P < 0.05.
sorghum tannin induced hypertrophy of the parotid glands; control and T/G groups, strongly suggesting that the tea group
the parotid glands in the tea group was double that in the
directly to tea.

The mechanism for these actions likely involves the binding of
gelatin to other proteins and chemical species such as iron.
Mehansho et al. (21) also reported that the inhibition of

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83–99.


3948–3952.


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proline-rich protein display a stronger affinity than with single proline-rich repeats.


