Long-term dose-response condensed tannin supplementation does not affect iron status or bioavailability

Nicole M. Delimont, Nicole M. Fiorentino, Katheryne A. Kimmel, Mark D. Haub, Sara K. Rosenkranz, Brian L. Lindshield*

Department of Food, Nutrition, Dietetics and Health, Kansas State University; Manhattan KS, USA.

*Correspondence: 208 Justin Hall, 1324 Lovers Lane, Manhattan KS 66506; blindsh@k-state.edu; Tel.: 785-532-7848

Abbreviations:

iAUC: incremental area under the curve

aPRP: acidic salivary proline rich protein

ASA24: automated system for 24-hour dietary recalls

BMI: body mass index

bPRP: basic proline rich proteins

CI: Confidence interval

CRP: C-reactive protein

EDTA: ethylene diaminetetraacetic acid

gPRP: glycosylated salivary proline rich protein

Hb: Hemoglobin

HPLC: High performance liquid chromatography

KSU: Kansas State University

PRP: salivary proline rich proteins
RDA: Recommended Dietary Allowance

TFA: Trifluoroacetic acid

USDA: United States Department of Agriculture

Conflict of interest: The authors declare no conflicts of interest.

Funding: United States Department of Agriculture (USDA) Foreign Agricultural Service under the Micronutrient Fortified Food Aid Products Pilot (MFFAPP) program, contract number #FFE-621-2012/033-00. Funding from this project enabled authors to publish open access.
Abstract

Background: Repeated phytic acid consumption leads to iron absorption adaptation, but the impact of repeated tannin consumption has not been established. Salivary proline-rich proteins (PRPs) may improve iron absorption by precipitating tannins.

Objective: To determine the effect of long-term, dose-response, condensed-tannin supplementation on iron bioavailability, and status as well as determine the effect of salivary proteins on iron bioavailability during prolonged condensed tannin consumption. A secondary objective was to assess astringency as a potential marker for adaptation to tannins and iron bioavailability.

Methods: Non-anemic women were enrolled in a double-blind three dose crossover trial (n = 11). Three (1.5, 0.25, or 0.03 g) condensed tannin supplements were consumed three times daily for four weeks in random order, with two-week washouts in-between. Before and after supplementation, meal challenges were employed to assess iron bioavailability, iron status, salivary PRP changes, and astringency.

Results: Tannin supplementation did not change iron bioavailability at any dose (ps > 0.82) from Week 0 to 4 in any dose. Hemoglobin (p = 0.126) and serum ferritin (p = 0.83) were unchanged by tannin dose from Week 0 to Week 4. There were significant correlations between iron bioavailability, basic PRPs (r = 0.366, p = 0.003), and cystatin production (r = 0.27, p = 0.03) with tannin supplementation. Astringency ratings did not change significantly within, or between tannin doses (ps > 0.126), but there were negative relationships between bPRP (rs < -0.32, ps < 0.21), cystatin (rs < -0.2, ps < 0.28) production, and astringency ratings.

Conclusions: Condensed-tannin consumption did not affect iron bioavailability or status regardless of supplementation period in premenopausal, non-anemic women. Correlation
analyses suggest that basic PRPs, and cystatins are associated with improved iron bioavailability, and that lower ratings of astringency may predict improved iron absorption with repeated tannin consumption.

Keywords: tannins, iron bioavailability, salivary proline rich proteins, adaptation, antinutritional factors, proanthocyanidins, iron deficiency anemia
Background

An estimated one billion people suffer from iron deficiency anemia worldwide (1). Most commonly, iron deficiency is found in women, children, vegetarians, and in people who have insufficient iron intake (1). Despite multiple initiatives aimed at reducing iron deficiency anemia in the past 20 years, an estimated 29% of non-pregnant women were anemic in 2011, a 4% reduction since 1995 (2).

Tannins have been found to negatively impact iron bioavailability (3-8) by formation of insoluble antinutritional-mineral complexes (9), and this has deterred tannin-rich foods, such as sorghum, from being used within food-aid for regions that are largely undernourished (10). Previous research suggests that long-term tannin consumption may not inhibit iron bioavailability as much as single meal studies predict (11, 12). For example, long-term antinutritional factor consumption in animals (13-15) and humans (16, 17) has resulted in improved non-heme iron bioavailability compared to single-meal studies. In these studies, the negative effects (reduced iron status or bioavailability) of antinutritional factor intake over time have not been sustained, and these findings have been proposed to be due to adaptation to antinutritional factors over time. In studies that have found reductions in iron bioavailability with tannin consumption, individual iron absorption has been highly variable (18, 19) and many individuals consuming diets with large concentrations of tannins maintain normal iron stores (20).

Many studies finding reduced iron bioavailability with tannin consumption have used hydrolyzable tannic acid, which is not commonly consumed, or tea tannins, which may be metabolized differently than condensed tannins, which are commonly found in food (11). To the best of our knowledge, no studies have determined the long-term effects of condensed tannin...
consumption apart from other antinutritional factors such as fiber or phytates on iron bioavailability or status. In addition, it has not been determined whether long-term condensed tannin consumption results in adaptation, or what mechanisms underlie adaptation if it does occur.

Mechanistically, adaptation to tannins may start in the mouth (21). Saliva contains six main classes of salivary proteins: histatins, cystatins, statherins, acidic proline-rich proteins (aPRP), basic proline-rich proteins (bPRP), and glycosylated proline-rich proteins (gPRP) that may exert independent effects on tannins (22). The binding of PRPs to condensed tannins also may prevent condensed tannins from chelating iron thereby improving iron bioavailability (21, 22). Tannin-PRP complexes are insoluble within the GI tract (23, 24), preventing tannin-iron chelation throughout digestion. There are a variety of PRP subtypes that make up different salivary profiles, which may be largely genetically determined (25, 26). Genetic determination of salivary profiles favoring effective tannin precipitation may explain why some individuals have greater capacity to consume tannins than others without negative impacts on iron status.

Upregulation of PRP secretion when consuming tannins has been shown to improve protein (27) and iron bioavailability in animal studies (12, 14), and animals that do not upregulate PRP synthesis in response to tannin consumption have poor growth outcomes (28).

Previously, PRP-tannin binding has been identified in sensory studies because it causes an oral astringency sensation (22). Theoretically, identification of PRP “adapters” may then be possible through simple, inexpensive, astringency testing (22). Further, changes in astringency sensation may indicate an upregulation of PRP production over time with repeated tannin consumption (29).
The primary objectives of the current tannin dose-response crossover trial were to determine the effect of 4-week, multi-meal dose-response condensed-tannin supplementation on iron bioavailability, and status, and to understand the effect of salivary proteins on iron bioavailability during prolonged condensed tannin consumption. A secondary objective was to assess astringency as a potential marker of adaptation to tannins and iron bioavailability.

In this study, we hypothesized that 1) condensed-tannin supplementation would not change iron bioavailability (determined by iron absorption) or status (determined by hemoglobin and ferritin) regardless of dose over four weeks, 2) salivary proline-rich protein production would be induced by tannin consumption over time and by higher versus lower tannin doses, and 3) proline-rich protein production would be positively associated with improved iron bioavailability after tannin consumption. Secondary hypotheses were that 1) astringency perception would be changed with tannin consumption over time, 2) salivary proline-rich protein production would predict astringency with tannin consumption, and 3) astringency could be used as a surrogate marker for iron bioavailability with tannin consumption.
Methods

Inclusion/exclusion

The study protocol was approved by the Institutional Review Board at Kansas State University (KSU; IRB #8121). Due to the length of the study, enrollment was rolling, and is outlined (Figure 1). An announcement requesting participants was sent to faculty and students through a university email digest, as well as disseminated through departmental social media channels. In total, 48 women responded, and potential participants were screened in person or via phone (Figure 1). Before screening, participants were required to read and sign an informed consent document, and all procedures, risks and benefits of the study were reviewed verbally. During screening, participants were asked to complete a medical history questionnaire.

Premenopausal women, aged 18-35, who were non-obese (body mass index, BMI ≤ 30.0 kg/m²), had no history of oral or gastrointestinal disease, were moderate (≤ 1 drink per day) or non-alcohol consumers, and non-tobacco users were eligible for participation. Non-anemic women were included who had both normal (n = 2, ferritin range 88-100 ng/mL) and marginal (n = 9, ferritin range 7-30 ng/mL) iron stores to minimize losses due to potential exacerbated anemia during the study. Iron absorption has been significantly changed after antinutritional supplementation in non-anemic, iron-replete individuals previously (6, 30). Further exclusion criteria included blood disorders affecting iron status or absorption, current supplementation or medication that would impair iron status, food allergies to supplements, pregnancy or breastfeeding. No participants consumed iron supplementation prior to, or during, the study period (see Supplementary Material for screening questionnaire and exclusion criteria).

Participants were compensated for completing study activities.

Study Design
Participants were assigned ID numbers, and a researcher not involved in data collection randomized each participant number to a tannin supplement order (SAS, Carey, NC). Participants, the principal investigator, and the project coordinator were blinded to dose order.

Supplementation periods consisted of Week 0 and Week 4 meal challenges, with four weeks of tannin supplementation in between (Figure 2). Four-week supplementation periods were chosen as completed previously (31) to assess for iron status changes with inhibited iron absorption at each meal, and to allow for time to adapt to tannins. Each participant consumed a powdered, condensed-tannin (Nusci grape seed extract, 95% condensed proanthocyanidins) supplement mixed in an opaque bottle with water and a non-caloric flavor enhancer and sweetener (Mio Original) to improve its palatability, three times daily for four weeks. Supplements were prepared weekly by an outside researcher; participants returned weekly to pick up supplements, and were questioned about supplement adherence. In addition, supplement bottles were checked for total supplement consumption, and adherence issues were noted. High (1.5 g), medium (0.25 g), or low (0.03 g) condensed-tannin doses were provided for consumption with three daily meals. These doses represented the amount of condensed tannins from 100g of high tannin red sorghum (32-34), 1 cup of tea (6-8), or the lowest inhibitory vegetable meals cited previously (3, 35). Each Week 4 meal challenge was followed with a two-week washout period to allow normalization of iron absorption and potentially PRPs to the participant’s usual diet (36). Two participants had a single washout period of three, instead of two weeks due to participant availability for meal challenges.
Tannin meal challenges

At Week 0 and Week 4 of each supplementation period, participants completed meal challenges at the KSU Physical Activity and Nutrition Clinical Research Consortium. Participants were asked to come in fasted (at least 8 hours) at 7:00 am, having abstained from teeth cleaning (2 hours) and exercise (24 hours) to minimize diurnal variations or other confounding factors in salivary production (37) and iron uptake (38, Figure 2b). Pre-meal saliva was collected by passive drool (2 ml total) in cryovials, and samples were immediately stored in a -80°C freezer. A 20-gauge indwelling peripheral IV catheter was placed either in the median cubital, cephalic, or basilic vein for multiple blood samples, which was flushed and saline locked (no intravenous fluids were provided during the meal challenges except for flushes) with 10 ml of 0.9% isotonic saline between blood collections. From the fasting blood draw, two separate samples were collected in 5 mL serum separator and 3 mL ethylenediaminetetraacetic acid (EDTA) vacutainer tubes to measure serum iron (by spectrophotometry), C-reactive protein (CRP, by nephelometry, sensitivity 0.2 mg/dL), ferritin (by immunoassay, sensitivity 0.1 ng/ml), and whole blood hemoglobin concentrations (by electronic cell cytometry). After fasted blood collection, a challenge meal including a 95 g bagel with 12 g sugar-free strawberry jam, half sprinkled with 15 mg anhydrous ferrous sulfate (39) and the other half with 75 mg ascorbic acid (16, 40), and a 90 g banana, was consumed simultaneously with the participant’s assigned supplement dose (1.5 g, 0.25 g, or 0.03 g in 8 fluid ounces). Ferrous sulfate and ascorbic acid weighed with a scale to the nanogram, and weights were recorded for %max iron calculations. Salivary samples were collected 15 minutes following the final bite of the meal to determine salivary protein stimulation after tannin consumption (41). Subsequent blood samples were
collected in tubes and analyzed for serum iron at 180 and 240 minutes. After collection, serum samples were centrifuged at 2500 RPM for 15 minutes after clotting for 20 minutes, and kept at room temperature for analysis. All blood samples were analyzed by a certified laboratory (Quest Diagnostics, Lenexa, KS) within 24 hours.

Regression analysis of three-point time draw for % maximum iron absorption

To minimize blood loss, validity of a three-time blood draw system to determine iron bioavailability by serum iron was determined. In the first supplementation period, four participants had blood drawn every thirty minutes for four hours to establish correlation with a three-time point draw (0, 180, and 210 minutes) system previously proposed (40). Regression of polynomial lines from data points were calculated on a computer data system (Microsoft Excel, 2013), and R^2 values were calculated for goodness-of-fit. From these data, it was verified that time points proposed were representative of the full models previously used (16, 40). Correlations of 0.99+ were seen, thus blood samples were also drawn for serum iron at these three time points to determine % max iron absorption and incremental serum iron area under the curve (iAUC) (40).

Incremental AUC and maximum percentage of iron absorption calculation

Serum iron data were used to calculate percentage of maximum iron recovery and iAUC for iron bioavailability analysis.

Percentage Iron Recovery: Percentage iron recovery was calculated as below (40).
\[
\% \text{ iron recovery max} = \left( \frac{\text{serum iron max} \times \text{plasma volume}}{\text{total iron ingested}} \right) \times 100
\]

Where:
\[
\text{iron max} = \frac{\mu \text{mol}}{L}, \text{plasma volume} = L, \text{and amount of iron is in } \mu \text{mol}
\]

\[
\text{plasma volume} (L) = \frac{\text{Blood volume (ml)} \times (1 - \text{Packed cell volume (decimal)})}{1000}
\]

And \( \text{blood volume} = 69.6 \frac{\text{ml}}{\text{kg body weight}} \)

\[\int \text{AUC} = \frac{\Delta \text{time}}{2} \times [\Delta \text{Serum iron}_{\text{time 0-180}} + (2 \times \Delta \text{Serum iron}_{\text{time 0-210}})]\]

**Astringency testing**

After the peripheral indwelling IV catheter was removed following each meal challenge, participants were asked to complete an astringency test (42, 43). Each participant was given four different concentrations of alum powder in 10 ml distilled water (0.20, 0.15, 0.07, and 0.03%) in random order to sip. They were first given a verbal description of the sensation of astringency and were asked to rate each solution based on their perception of astringency on a 5 point Likert scale (1 = not astringent, 5 = extremely astringent). Participants waited for 30 seconds before testing the next sample.

**Dietary analysis in supplementation periods**

Within each supplementation period, 24-hour dietary recalls were collected on three different days (two weekdays and one weekend day, 44). At the beginning of week two of each four-week supplementation period, participants were emailed a unique username and password.
to complete 24-hour dietary recalls for 2 weekdays and 1 weekend day on the automated Self-Administered 24-Hour Recall (ASA24®). After all recalls were collected in each supplementation period, dietary data were extracted, and total caloric intake (kcal), protein (g), fat (g), carbohydrate (g), iron (mg), ascorbic acid (mg), meat protein (oz.), sugar (g), fiber (g), Zn (mg), and Cu (mg) content were averaged from system calculated amounts for each participant.

Food intake logs were downloaded from the ASA24® for manual calculation of proanthocyanidins and polyphenols. During this process, a research assistant reviewed all dietary data for each participant using an electronic spreadsheet (Microsoft Excel). Food items were referenced from United States Department of Agriculture (USDA) tables pulled into an electronic spreadsheet, and total proanthocyanidin (condensed tannin, 45) and polyphenol (46) amounts were calculated and summated for each recall. From these summations, averages were calculated. All assessments and calculations were reviewed by the project coordinator before analyses were completed.

Salivary PRP measurement

Acidified saliva sample preparation

Frozen salivary samples were thawed overnight in a refrigerator. Before sample analysis, consistency in chromatogram output with duplicate samples was verified, and samples were analyzed in a single run. For PRP extraction, 900 µl of saliva was mixed with 10 µl of 10% trifluoroacetic acid (TFA) in water, centrifuged for 5 min at 8000 RPM, and the supernatant was filtered through a 0.2 µm PVDF syringe filter as described previously (47). Before samples were analyzed, it was verified that there was no PRP peak loss with use of syringe filters by testing in
trial HPLC runs. The supernatant was then analyzed by high performance liquid chromatography (HPLC).

**HPLC parameters and equipment**

All reagents were analytical grade. Acetonitrile, TFA, and HPLC grade water were purchased from Fisher Scientific. Ninety µl of salivary supernatant was injected into a Fisher 2.1x150 mm, 5µm BioBasic C8 analytical column at a flow rate of 0.3 ml/min for 49 minutes at 40°C with an autosampler (Shimadzu SIL) on a HPLC system containing a LC20AB pump (Shimadzu), and a Shimadzu SPD-M20A PDA system. Detection of PRPs was carried out at 214 nm (47, 48). Mobile phase consisted of 0.2% TFA in HPLC grade water (A) and 0.2% TFA in 80/20 acetonitrile and HPLC grade water (B) (41, 47-49). A linear gradient was applied from 0-39 minutes from 0-54% (B), then from 39-49 minutes at 54-100% B to elute late proteins (41, 47-49). After each run, the column was washed and stabilized with initial conditions by increasing linear gradient back to 100% A over 10 minutes.

**Statistical analyses**

Data were analyzed using SAS statistical software (SAS Studio version 3.6, Cary, North Carolina), statistical significance was set at $p < 0.05$. All data are presented as mean ± standard deviation (SD). Before analysis, all data were analyzed for normality and homogeneity of data in Q-Q plots and with Levene’s tests. Variables that were non-normal (proanthocyanidin monomers, dimers, total proanthocyanidin, ascorbic acid, sugar, and iron intake) were log transformed, and determined to be normal before further analysis. Log-transformed variables
were included in stepwise variable selection in adjusted model building (below). All log-
transformed data were back-transformed for results presentation.

Sample size

A paired t-test sample size calculation (SAS studio version 3.6, Cary, NC) determined
that four participants would be needed to detect a change in iAUC of 41%, which was observed
in a similarly designed antinutritional factor adaptation study (16) as statistically significant with
80% power and at an α-level of 0.05.

Demographic data, washout, and randomization order analysis

Week 0 demographic and nutritional intake data were analyzed by analysis of variance by
supplementation period. Randomization order and previous dose effect were analyzed by Chi-
square testing to assess for bias in supplementation period order, or previous effect of
supplementation period. Changes between previous and next supplementation period during
washout were analyzed for hemoglobin, ferritin, iAUC for serum iron, and % max iron
absorption by analysis of variance.

Hematological outcomes analysis

Regression analysis of hematological outcomes

Linear regression of raw outcomes data was used to determine whether four weeks of
multiple daily tannin supplementation would change iron absorption or status within or between
supplementation periods. In regression analysis, differences between supplementation periods
were analyzed for % max iron absorption, ferritin, and hemoglobin at Weeks 0 and 4 (to analyze for within dose responses). Multiple regression was used to adjust models for repeated (participant) and random (ferritin, CRP, dietary intake, weight, and age) covariates after stepwise selection for significant (p < 0.05) variables. To maximize analysis of individual iron bioavailability and status within different supplementation periods, individual movements (increase, decrease, or maintain) in dose-responses (hemoglobin, ferritin, % max iron absorption, and serum iron iAUC) were analyzed with Chi-square testing and Fisher’s exact tests.

PRP and astringency outcomes analysis

PRP changes with tannin supplementation and correlations with iron bioavailability

To determine whether salivary PRP production would be inducible by tannin consumption both over time and in a dose-dependent manner, salivary proteins were divided into type by retention times (41, 47-49), peak mAu were recorded for each, and protein subtypes were aggregated to quantify total salivary proteins and PRPs. Salivary protein subtypes were further analyzed by proportion to total mAu from the equation:

\[
PRP \text{ subtype proportion} = \frac{PRP \text{ type (area sum)}}{Total \ PRP \ area}
\]

Or

\[
Total \ PRPs = \frac{Total \ PRP \ area}{Total \ salivary \ protein \ area}
\]
Differences in salivary protein production from Week 0 to 4 within doses were analyzed by multiple factor analysis of variance. To determine whether PRP production would impact iron bioavailability with tannin consumption, Pearson’s product-moment correlations were used to determine correlations between % max iron absorption, iAUC for serum iron, randomization order, and PRP types.

Astringency perception, connections to salivary protein production, and iron bioavailability

We determined whether astringency perception was changed within or between tannin doses using Chi-square testing and Fisher’s exact tests by allocated, and previous dose. Connections between salivary protein production, iron bioavailability and astringency were analyzed by Pearson’s product-moment correlations.

Results

Week 0 demographics

Mean participant age was 26 ± 1.2 yrs, and ranged from 20-35. All participants were occasional (2-3 drinks/month) or moderate (2-3 drinks/week) alcohol consumers. Aside from one participant, who consumed a vegan diet, and took vitamin B₁₂ supplements, no participants took vitamin or mineral supplements during the study period. The average BMI of participants was 24 ± 2.4 kg/m² (range 18.2-28.9). Participant weights (kg) did not significantly change between tannin doses nor from Week 0 to Week 4 of each supplementation period.

Supplementation order and outcomes measures
With our randomization procedure, six of the eleven participants were randomized to 1.5 g tannin doses during the first supplementation period, and 0.03 g doses during the second supplementation period. Incremental AUC for serum iron ($p = 0.118$), hemoglobin ($p = 0.87$), and ferritin ($p = 0.15$) were not different by order of tannin dose in any supplementation period. Supplementation order did significantly positively impact % max iron absorption following the 1.5 g tannin dose when taken in the third versus the first supplementation order ($p = 0.046$), meaning that lower doses taken before the 1.5 g dose led to significantly improved iron bioavailability during that intervention period. There were no significant differences in Week 0 to Week 4 dose-responses for hemoglobin, ferritin, serum iron iAUC, or % max iron absorption when accounting for previous dose by Chi-square testing (results shown in Supplementary Material). There were no significant changes in hemoglobin ($p = 0.993$), ferritin ($p = 0.982$), iAUC for serum iron ($p = 0.984$), or % max iron absorption ($p = 0.998$) at each Week 0-time point, and previous tannin dose did not affect outcomes changes during washout for hemoglobin ($p = 0.68$), ferritin ($p = 0.511$), % max iron absorption ($p = 0.735$), or iAUC for serum iron ($p = 0.137$). No salivary protein measurements were significantly correlated with tannin dose order ($ps > 0.62$).

**Study dietary intake**

There was wide variability in nutrient consumption during supplementation periods, but there were no significant differences in total calorie, macronutrient, meat, fiber, or micronutrient consumption between tannin doses (Table 1). Despite wide variability in nutrient consumption, individual macronutrient and micronutrient intake were not different between tannin doses. Iron intake was 7-18% less than the recommended dietary allowance (RDA) of 18 mg in all
supplementation periods, ascorbic acid exceeded the RDA by 15-80%. While not significant, dietary proanthocyanidin intake (apart from supplements) trended towards lower amounts in the 0.03 g (69.1 ± 78.9 mg) and 0.25 g (82.3 ± 85.1 mg) doses compared to the 1.5 g dose (123.2 ± 136.6 mg; \( p_s > 0.09 \)). On average, 0.03 g, 0.25 g, and 1.5 g tannin supplements constituted 2, 8, and 35-fold the average dietary proanthocyanidin intake for their respective supplementation period.

**Supplementation period iron absorption, hematological indices of iron status**

*Unadjusted regression outcomes*

Individual level data are included in the Supplementary Material. There were no changes in unadjusted iron bioavailability (by iAUC and % max iron absorption) within, or between, tannin supplementation periods (Table 2). In addition, there were no differences at Week 0 (\( p = 0.82 \)) or Week 4 (\( p = 0.92 \)) unadjusted serum iron iAUC or Week 0 (\( p = 0.82 \)) or Week 4 (\( p = 0.62 \), Table 2) % max iron absorption between tannin doses. Hemoglobin and ferritin values were not different at Week 0 or Week 4 for any tannin dose (Hb: Week 0: \( p = 0.838 \); Week 4: \( p = 0.68 \) and ferritin: Week 0: \( p = 0.855 \); Week 4 \( p = 0.575 \), Table 2). There were no significant differences in hemoglobin (\( p = 0.90 \)), ferritin (\( p = 0.81 \)), % max iron absorption (\( p = 0.39 \)), or serum iron iAUC (\( p = 1.0 \)) for improvement, deterioration, or maintenance by any tannin dose through Chi-square testing (Table 3).

*Stepwise linear regression analysis and adjusted regression models*

To test the impact of dietary and individual physiological differences (iron status, anthropometric, salivary protein) on iron bioavailability and status, we employed stepwise
regression analysis to establish significant covariates to build an adjusted model for
hematological outcomes. Covariates that were significantly positively associated with serum iron
iAUC and % max iron absorption included bPRP and cystatin production (Table 4). Significant
covariates that were negatively associated with serum iron iAUC and % max iron absorption
included aPRP and total salivary protein production, higher rating of 0.2 mg/dL astringency
testing, and total meat consumption. Significant covariates positively associated with ferritin
levels included bPRP production, and zinc consumption.

Significant covariates for each outcome measure were added to the linear regression for
adjusted outcomes analysis. Following the full adjustment for significant covariates, neither
serum iron iAUC or % max iron absorption was statistically different between, or within, each
tannin dose (Table 4, Figures 3, 4). There were no significant differences in adjusted
hemoglobin or ferritin values within, or between, tannin supplementation periods (Table 4).

Correlations between salivary protein production and iron absorption with tannin
supplementation

There were no significant correlations between total salivary protein production and iron
absorption (by % max iron absorption and iAUC for serum iron) during the study. In all tannin-
doses, and when combining all data from 4-week supplementation periods, bPRP production was
significantly and positively correlated with % max iron absorption at Week 0 and Week 4 (Table
5). There were more positive correlations with Week 4 0.03 and 0.25 g dose-max iron absorption
% and bPRP production than the 1.5 g dose (Table 5), suggesting that bPRP production was
potentially important to enhance iron bioavailability for lower, but not higher tannin doses. Week
0 and Week 4 aPRP production was significantly negatively correlated with iron absorption in
each supplementation period (Table 5). Total gPRP production was significantly negatively
correlated with iron bioavailability at Week 4 in the 1.5 g supplementation period (Table 5).  
Statherin production was non-correlated with iron absorption, while cystatin was overall
significantly positively correlated with iron absorption (Table 5).

**Astringency testing**

**Astringency ratings with tannin consumption**

Astringency ratings did not change among participants with changes in tannin doses, and
were not statistically affected by order of tannin dose in Chi-square testing ($p_s > 0.09$), except for
the lowest astringency doses, which were rated significantly lower after 1.5 g tannin doses ($p =
0.047$; Table 6). There were no significant effects of tannin dose on changes in ratings of
astringency ($p_s > 0.126$), however, overall ratings of astringency were lower for the 0.15 mg/dL
astringency dose due to supplementation with the 1.5 g tannin dose compared to the 0.03 g
tannin dose ($p = 0.013$).

**Astringency ratings with PRP production**

Cystatin and bPRP production were correlated with lower astringency sensation in all
alum doses. In correlations from individual participants with astringency ratings at the highest
alum concentration (0.2 mg/dL), there were significant, positive correlations between total
salivary proteins (7 of 11 participants, $r > 0.49, p < 0.05$) and astringency, and negative
relationships between bPRPs (9 of 11 participants, $r = -0.32$ to $-0.81, p = 0.001 - 0.21$), cystatins
(9 of 11 participants, $r = -0.2$ to $-0.76, p = 0.03 - 0.28$) and astringency.
The primary objectives of this trial were to determine the effect of long term dose-response condensed-tannin supplementation on iron bioavailability, and status, and to understand the effect of salivary proteins on iron bioavailability during prolonged condensed tannin consumption. Secondarily, the study assessed astringency as a potential marker for adaptation to tannins and iron bioavailability.

**Hematological outcomes and tannin supplementation periods**

Overall, our results support the hypotheses of no significant reductions in iron bioavailability or status with three supplementation periods of long-term, multiple-daily tannin supplements over four weeks. Despite non-significant negative trends in Week 0 iron absorption with 1.5 g (highest) compared to 0.25 and 0.03 mg (lowest) doses, hemoglobin and ferritin were maintained in all groups throughout supplementation periods (Table 2). There were no differences in ferritin or CRP measurements within individuals, or among tannin doses throughout the study (Table 2). To our knowledge, this is the first study that has quantified effects of long-term, dose-response condensed tannin effects on iron bioavailability and status. Our findings of no significant changes in iron bioavailability or status within or among tannin doses are contrary to previous single meal studies using black tea (6, 50, 51), which contain theaflavins and thearubigins (52), or in trials using tannic acid (3, 4, 51). Tannic acid and tea tannins may bind to salivary proteins and chelate iron differently than condensed tannins (proanthycyanidins), which are typically larger in size and consumed within a complex food.
matrix (53, 54). Condensed tannin models in humans and rats (55-58), also found no changes in iron bioavailability or status with tannin consumption over time are consistent. In contrast, dose-dependent inhibition of grape seed extract on iron bioavailability has been reported in Caco-2 cells (59). There have been similar discrepancies in \textit{in vivo} and \textit{in vitro} models cited previously. Iron status in pigs consuming red (higher tannins) and white beans (lower tannins) resulted in no difference in iron status outcomes, while the Caco-2 cell model found higher iron bioavailability from white than red beans (60). Inconsistencies between long-term in vivo and \textit{in vitro} studies may be partially a result of the complexity of factors contributing to human and animal digestion, including salivary proteins, which likely are not accounted for in simulated digestion. In addition, the single-meal digestion simulation used in Caco-2 cells might have the same limitations as short-term bioavailability studies. The discrepancies between long-term consumption studies compared to short-term bioavailability studies and Caco-2 findings may suggest that caution needs to be exercised when using the evidence from the latter types of research to predict chronic consumption \textit{in vivo} iron outcomes.

The current study is the first that we know of that has quantified the effects of multiple daily, multi-dose condensed proanthocyanidins on iron bioavailability or status. A similar study found that 4-week tea supplementation, similar to this study’s 0.25 g supplement dose, resulted in significantly lower ferritin levels in non-anemic and anemic women (31), suggesting that there may be differences in the impact of tannin-type on iron status. Interestingly, in a study observing effects from green leafy vegetables on hemoglobin, significant improvements were seen after only three weeks (61). While supplementation levels in the present study at 1.5 g were 50 x greater than 0.03 g and 10 x greater than 0.25 g tannin doses, iron absorption was only modestly reduced in the 1.5 g supplementation period (equivalent to consuming 100g of high tannin
sorghum three times daily), and there were no significant reductions in iron status over time. There were no changes in iron absorption, ferritin, or hemoglobin over time in any adjusted or unadjusted models, suggesting that condensed tannin intake at any dose did not affect iron absorption. While other studies have noted reduction in iron bioavailability with condensed tannin intake (4, 62), the current study is the first that we know of to isolate supplementation of proanthocyanidins outside of other antinutritional factors commonly consumed concurrently in vivo.

**PRP production and iron bioavailability**

The current study is the first, to the best of our knowledge, that has assessed correlations between salivary protein production and iron bioavailability, or investigated long-term tannin supplementation effects on salivary profiles in humans. Overall, our hypotheses that salivary PRP production would be inducible in higher compared to lower tannin doses, and that PRP production would impact iron bioavailability with tannin consumption were partially supported. There were not significant changes in PRP or salivary protein production within or among tannin doses, however, there were significant correlations between PRPs, non-PRP salivary proteins, and iron bioavailability, suggesting that participants producing higher quantities of total salivary proteins, bPRPs, and cystatins improved iron absorption with condensed tannin intake. Correlations between bPRPs, cystatins, and iron absorption tended to be stronger at Week 4 in lower doses, suggesting that salivary protein subtypes may change with regular tannin consumption to improve iron bioavailability, but are not likely the only physiological adaptation when consuming higher tannin doses. In caco-2 cells, bPRPs have been found to inhibit uptake of small tannin molecules through formation of insoluble complexes, but this process was
mediated in part by sodium-glucose transporter-1 (SGLT-1) and multidrug resistance protein (MRP2) (63). It may be that bPRPs signal changes in these receptors that mediate tannin absorption and iron related sequelae.

Binding of bPRP to polyphenols may be preferential versus other PRP subtypes (64), and production of larger bPRPs that would efficiently bind to tannins are most likely genetically determined (64-66). This idea may help to explain the wide variability in iron absorption among participants and age-related changes in iron absorption with tannin consumption. For example, in pre-term infants, salivary protein profiles vary widely from adults (49), and bPRPs are almost non-existent, which may affect tolerance of the former population to tannins.

Contrary to findings that bPRPs supported iron bioavailability with tannin consumption, gPRP and aPRP production, especially at Week 0 for each tannin dose, were significantly negatively correlated with iron bioavailability. Negative impacts of these PRP subtypes on iron bioavailability could mean that individuals producing higher levels of aPRP or gPRP proteins less efficiently absorb iron, especially when initially exposed to increased concentrations of tannins and until other homeostatic protective mechanisms are employed. This is the first time, to the best of our knowledge, that aPRP and gPRP interactions with tannins over time have been determined in vivo. It may be that aPRP and gPRP are upregulated with tannin consumption, but do not bind to condensed tannins effectively, thereby increasing protein-iron chelation. Further, aPRP and gPRP may be effectively inhibited by carbohydrate consumption (67) compared to bPRP, meaning that individuals producing more of these proteins may less effectively prevent tannin-iron chelation.

**Astringency as a predictor of iron bioavailability with tannin consumption**
Our secondary hypotheses that: 1) astringency perception would be changed with tannin consumption over time, 2) salivary proline-rich protein production would predict astringency, and 3) astringency could be used as a surrogate marker for iron bioavailability, based on PRP expression with consumption were partially supported by our findings. Astringency ratings did not change within or among tannin doses throughout the study, except for the highest (1.5g) tannin dose. Astringency ratings were lower with higher tannin concentrations, and were also significantly negatively correlated with bPRP and cystatin production, suggesting that reductions in ratings of very astringent, or bitter foods, may help predict iron bioavailability with tannin exposure. Despite this, we did not find consistent associations between iron bioavailability and astringency ratings within or between supplementation periods.

Limitations

There are several important limitations that must be considered when interpreting results from the current study. It must be acknowledged that tannin-supplementation limits the generalizability of these findings to tannins within foods, which commonly co-exist with other antinutritional factors, such as phytic acid. Tannin-rich food commodities may also confer different effects with antinutritional-food matrix interactions. Beyond tannin supplement limitations themselves, several factors, including above RDA ascorbic acid intake, and challenge meal ascorbic acid supplementation may have inhibited tannin effects on iron bioavailability as seen previously (62), although iron bioavailability has been inhibited with similar doses of ascorbic acid in test meals elsewhere (16). In addition, the population assessed in this study consisted of non-anemic, pre-menopausal adult women with a sufficient and varied diet. Given
that women were non-anemic, and Week 0 ferritin stores ranged from 7-100 ng/mL, it is possible that 4 weeks may not have been long enough for multimeal supplementation to impact iron status, however antinutritional factors have been shown to change serum ferritin and hemoglobin in as little as 2-4 weeks (31, 68-71). Iron bioavailability in our study was less than the 9% suggested previously for the model employed here (40), and although the present study findings are consistent with many studies testing effects of antinutritional factors (3-8, 16, 40), low iron bioavailability may have impacted the sensitivity of iron absorption curves between supplements (40). Similarly, variability in iron absorption limited the power of our sample size, and may have diminished the small impact of tannins on iron absorption observed here. Despite this, it is important to consider the lack of concentration-dependent effect from tannins on individual study participants that had limited variability in iron absorption throughout the supplementation periods. Individual results from crossover design support our findings overall (Supplemental materials). It may be problematic to generalize these findings to a clinical population, for example, anemic women and children, who may have a different response to tannin exposure.

Participants noted that they experienced increased salivary flow rates during 1.5 g compared to 0.03 tannin doses, although flow rates were not measured quantitatively. It is also important to note that while concentrations of PRPs themselves did not change through the study, subjective experiences of salivary flow rates among participants was greater at Week 4 in higher, 0.25 and 1.5 g, supplementation periods than at Week 0. Previous research findings have indicated that salivary flow along with PRP concentration have given more accurate estimates of total production than concentration alone (72). Salivary flow rate has been found to be an independent factor in reducing ratings of astringency along with salivary protein concentration
suggesting that in our study, total PRP production may have been increased with increasing salivary flow rates. Lack of measurement of salivary flow rate is a limitation in PRP-iron outcomes analysis because we were not able to assimilate total PRP quantification from a pre-determined 2 ml salivary sample (which was obtained over varying time spans). Lastly, we grouped salivary types based on elution times. This has been previously employed (47), but is not an accurate representation of salivary protein quantification.

**Future directions**

Foremost, better characterization of proanthocyanidin-phytic acid interactions on iron bioavailability and salivary protein production needs to be explored, including the effects of mixed antinutritional factor outcomes regarding iron bioavailability over time. Mixed diets have conferred different findings in the past (74) than those presented in the current research study, and understanding nutrient interactions may be key to understanding these discrepancies. In addition, effects of tannins in anemic populations, who may have disease burden or dietary deficiencies, need to be explored.

Due to the complexity of PRP subtypes, determining which specific bPRP and cystatins improve iron bioavailability with tannin challenge may enable diet-specification in both children and adults (64). Determination of PRP genetic makeup in anemic and non-anemic tannin consumers may help to determine those with tannin-binding subtypes, and protein production could later be determined based on findings. More studies are needed to determine the effects of tannin supplementation on iron bioavailability in infants, and the effects of different tannin types (tannic acid, theaflavins and thearubigins) on salivary proteins. Further comparison of oral and
enteric tannin exposure may help to determine non-salivary determinants of physiological tannin mediation.
Conclusions

Long-term condensed tannin supplementation did not impair iron bioavailability, ferritin, or hemoglobin levels in non-anemic, premenopausal women. Iron absorption following tannin supplementation was positively correlated with bPRP and cystatin production, and tannin supplementation was associated with significantly reduced ratings of astringency over time. These findings suggest that individual physiology may need to be accounted for when considering nutritional impact on iron bioavailability and status. Given the lack of impact of condensed tannins on iron status over time, these results suggest that efforts to remove condensed tannins from the diet to increase iron bioavailability and status may need to be reconsidered.

Acknowledgements: Thank you to the KSU Physical Activity and Nutrition Clinical Research Consortium for use of facilities during the study.

Author Contributions ND conceived and conducted experiments, analyzed data, and wrote the manuscript. NF randomized participants to supplementation periods, prepared supplements throughout the study, and edited the manuscript. KK analyzed salivary proteins on HPLC and edited the manuscript. SR conceived the experiment and edited the manuscript, MH conceived and edited the manuscript. BL conceived and oversaw the experiment, analyzed data, and edited the manuscript.
References


57. Hamdaoui MH, Chabchoub S, Hedhili A. Iron bioavailability and weight gains to iron-deficient rats fed a commonly consumed Tunisian meal 'bean seeds ragout' with or without beef and with green or black tea decoction. *J Trace Elem Med Biol.* 2003;17:159-64.


Tables

Table 1: Dietary intake of calories, macronutrients, micronutrients, and proanthocyanidins by supplementation period

<table>
<thead>
<tr>
<th>Supplementation period</th>
<th>0.03 g</th>
<th>0.25 g</th>
<th>1.5 g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>N</td>
<td>11</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Kcal/day</td>
<td>2186.2 ± 570.9</td>
<td>2230.5 ± 640.6</td>
<td>1957.8 ± 348</td>
</tr>
<tr>
<td>Protein (g/day)</td>
<td>80.8 ± 27.2</td>
<td>79.7 ± 21.6</td>
<td>71.6 ± 16.6</td>
</tr>
<tr>
<td>Fat (g/day)</td>
<td>90.3 ± 30</td>
<td>93.5 ± 27.9</td>
<td>71.1 ± 19.5</td>
</tr>
<tr>
<td>Carbohydrates (g/day)</td>
<td>259.3 ± 96.2</td>
<td>268.4 ± 109.9</td>
<td>252.1 ± 126.2</td>
</tr>
<tr>
<td>Meat (oz/day)</td>
<td>3.61 ± 2.5</td>
<td>3.81 ± 2.6</td>
<td>3.6 ± 1.9</td>
</tr>
<tr>
<td>Sugar (g/day)</td>
<td>122.8 ± 57.9</td>
<td>127.7 ± 69.3</td>
<td>120.0 ± 99.8</td>
</tr>
<tr>
<td>Fiber (g/day)</td>
<td>21.2 ± 12</td>
<td>19.2 ± 10</td>
<td>21.6 ± 14.3</td>
</tr>
<tr>
<td>Iron (mg/day)</td>
<td>15.1 ± 6.6</td>
<td>15.7 ± 7.2</td>
<td>14.7 ± 6.2</td>
</tr>
<tr>
<td>Ascorbic acid (mg/day)</td>
<td>109.7 ± 87.9</td>
<td>80.9 ± 66.9</td>
<td>110.4 ± 142.8</td>
</tr>
<tr>
<td>Zinc (mg/day)</td>
<td>12.6 ± 5</td>
<td>12.8 ± 5.6</td>
<td>10.2 ± 2.5</td>
</tr>
<tr>
<td>Copper (mg/day)</td>
<td>1.5 ± 0.69</td>
<td>1.5 ± 0.91</td>
<td>1.4 ± 0.61</td>
</tr>
<tr>
<td>Monomers (mg/day)</td>
<td>8.0 ± 7.5</td>
<td>18.2 ± 23.4</td>
<td>16.1 ± 21.8</td>
</tr>
<tr>
<td>Dimers (mg/day)</td>
<td>8.6 ± 6.6</td>
<td>13.5 ± 15.7</td>
<td>14.8 ± 17.3</td>
</tr>
<tr>
<td>Trimers (mg/day)</td>
<td>5.3 ± 4.5</td>
<td>6.7 ± 8.0</td>
<td>8.9 ± 12.5</td>
</tr>
<tr>
<td>4-6 mers (mg/day)</td>
<td>15 ± 16.3</td>
<td>16.6 ± 19.4</td>
<td>27.5 ± 36.3</td>
</tr>
<tr>
<td>7-10 mers (mg/day)</td>
<td>9.6 ± 12.7</td>
<td>9.0 ± 9.8</td>
<td>15.7 ± 18.7</td>
</tr>
<tr>
<td>Polymers (mg/day)</td>
<td>22.7 ± 39.5</td>
<td>18.4 ± 25.7</td>
<td>40.3 ± 51.3</td>
</tr>
<tr>
<td></td>
<td>Total proanthocyanidin (mg/day)</td>
<td>Total polyphenol intake (mg/day)</td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>---------------------------------</td>
<td>---------------------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>69.1 ± 78.9</td>
<td>1106.6 ± 531.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>82.3 ± 85.1</td>
<td>1139.6 ± 647.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>123.2 ± 136.6</td>
<td>1108.9 ± 590</td>
<td></td>
</tr>
</tbody>
</table>

SD: standard deviation,

No significant differences ($p > 0.05$)
Table 2: Unadjusted iron bioavailability, status, and inflammatory markers at Week 0 and Week 4 of each supplementation period

<table>
<thead>
<tr>
<th></th>
<th>Week 0</th>
<th>Week 4</th>
<th>Week 0</th>
<th>Week 4</th>
<th>Week 0</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Mean</td>
<td>Mean</td>
<td>Mean</td>
<td>Mean</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>95% CI</td>
<td>95% CI</td>
<td>95% CI</td>
<td>95% CI</td>
<td>95% CI</td>
</tr>
<tr>
<td>% Max iron</td>
<td>12.7</td>
<td>10.7</td>
<td>12.1</td>
<td>12.4</td>
<td>11.2</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>(7.5, 17.9)</td>
<td>(5.4, 15.9)</td>
<td>(6.9, 17.3)</td>
<td>(7.2, 17.6)</td>
<td>(6.0, 16.5)</td>
<td>(5.0, 15.5)</td>
</tr>
<tr>
<td>iAUC for serum iron (µg/dL*hr)</td>
<td>2155</td>
<td>2269</td>
<td>2461</td>
<td>2769</td>
<td>2237</td>
<td>2277</td>
</tr>
<tr>
<td></td>
<td>(612, 3696)</td>
<td>(727, 3810)</td>
<td>(919, 4003)</td>
<td>(1228, 4311)</td>
<td>(696, 3779)</td>
<td>(735, 3819)</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>13.2</td>
<td>13.3</td>
<td>13.3</td>
<td>13.4</td>
<td>13.4</td>
<td>13.3</td>
</tr>
<tr>
<td>Ferritin (ng/ml)</td>
<td>35.4</td>
<td>42.3</td>
<td>35.8</td>
<td>37.3</td>
<td>40.0</td>
<td>44.5</td>
</tr>
<tr>
<td></td>
<td>(28.4, 42.4)</td>
<td>(35.3, 49.3)</td>
<td>(28.8, 42.8)</td>
<td>(30.3, 44.3)</td>
<td>(33.0, 47.0)</td>
<td>(37.5, 51.5)</td>
</tr>
<tr>
<td>CRP (mg/dL)</td>
<td>0.2</td>
<td>0.3</td>
<td>0.3</td>
<td>0.2</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>(0.0, 0.5)</td>
<td>(0.0, 0.5)</td>
<td>(0.1, 0.5)</td>
<td>(0.0, 0.4)</td>
<td>(0.0, 0.4)</td>
<td>(0.1, 0.6)</td>
</tr>
</tbody>
</table>

CI: confidence interval, iAUC: incremental area under the curve; CRP: C-reactive protein.

No significant differences ($p > 0.05$).
Table 3: Comparison of improvement, maintenance, or deterioration of iron bioavailability and status within each supplementation period

<table>
<thead>
<tr>
<th>Supplementation period</th>
<th>Improvement</th>
<th>Maintenance</th>
<th>Deterioration</th>
<th>Fisher’s exact (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% max iron absorption</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.03 g</td>
<td>5</td>
<td>0</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>0.25 g</td>
<td>8</td>
<td>0</td>
<td>3</td>
<td>0.394</td>
</tr>
<tr>
<td>1.5 g</td>
<td>5</td>
<td>0</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>0</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>iAUC serum iron µg/dL*hr</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.03 g</td>
<td>6</td>
<td>0</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>0.25 g</td>
<td>6</td>
<td>0</td>
<td>5</td>
<td>1.0</td>
</tr>
<tr>
<td>1.5 g</td>
<td>6</td>
<td>0</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>0</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.03 g</td>
<td>6</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>0.25 g</td>
<td>5</td>
<td>2</td>
<td>4</td>
<td>0.896</td>
</tr>
<tr>
<td>1.5 g</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>8</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Ferritin (ng/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.03 g</td>
<td>7</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>0.25 g</td>
<td>5</td>
<td>1</td>
<td>5</td>
<td>0.816</td>
</tr>
<tr>
<td>1.5 g</td>
<td>6</td>
<td>0</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>2</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

iAUC: incremental area under the curve

No significant differences: \( p < 0.05 \)
Table 4: Estimation of iron bioavailability and status due to supplementation period, time, and significant covariates

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>SE B</th>
<th>β</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>6.92</td>
<td>&lt;0.0001</td>
<td>1.5</td>
<td>0.81</td>
<td>0.82</td>
</tr>
<tr>
<td>Constant</td>
<td>48.9</td>
<td>22.7</td>
<td>0</td>
<td>2.16</td>
<td>0.004</td>
</tr>
<tr>
<td>Supplementation period</td>
<td>-0.36</td>
<td>1.5</td>
<td>-0.023</td>
<td>0.81</td>
<td>0.82</td>
</tr>
<tr>
<td>% max iron absorption</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>-4.2</td>
<td>1.7</td>
<td>-0.20</td>
<td>-1.54</td>
<td>0.034</td>
</tr>
<tr>
<td>Ferritin</td>
<td>-0.06</td>
<td>0.03</td>
<td>-0.23</td>
<td>-2.00</td>
<td>0.023</td>
</tr>
<tr>
<td>CRP</td>
<td>-26.1</td>
<td>3.7</td>
<td>-0.74</td>
<td>-5.91</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>bPRP</td>
<td>10.22</td>
<td>3.02</td>
<td>0.25</td>
<td>2.00</td>
<td>0.023</td>
</tr>
<tr>
<td>aPRP</td>
<td>-16.9</td>
<td>6.5</td>
<td>-0.21</td>
<td>1.86</td>
<td>0.012</td>
</tr>
<tr>
<td>Cystatin</td>
<td>0.0016</td>
<td>0.0004</td>
<td>0.06</td>
<td>2.51</td>
<td>0.0008</td>
</tr>
<tr>
<td>Model</td>
<td>9.81</td>
<td>&lt;0.0001</td>
<td>1.5</td>
<td>0.81</td>
<td>0.82</td>
</tr>
<tr>
<td>Constant</td>
<td>10281</td>
<td>1869.2</td>
<td>0</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>Supplementation period</td>
<td>65.94</td>
<td>299.6</td>
<td>0.022</td>
<td>0.22</td>
<td>0.83</td>
</tr>
<tr>
<td>iAUC serum iron</td>
<td>-210.6</td>
<td>379.4</td>
<td>-0.05</td>
<td>-0.56</td>
<td>0.58</td>
</tr>
<tr>
<td>CRP</td>
<td>-2091.3</td>
<td>514.5</td>
<td>-0.40</td>
<td>-4.06</td>
<td>0.0002</td>
</tr>
<tr>
<td>μg/dL*hr</td>
<td>0.0042</td>
<td>0.001</td>
<td>0.31</td>
<td>3.12</td>
<td>0.003</td>
</tr>
<tr>
<td>Total salivary Protein</td>
<td>-6606</td>
<td>1731</td>
<td>-0.38</td>
<td>-3.82</td>
<td>0.0004</td>
</tr>
<tr>
<td>Meat</td>
<td>-185.7</td>
<td>78.3</td>
<td>-0.23</td>
<td>-2.37</td>
<td>0.022</td>
</tr>
<tr>
<td>0.2 astringency</td>
<td>-237.3</td>
<td>238.4</td>
<td>-0.31</td>
<td>-2.83</td>
<td>0.0066</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>Model</td>
<td>5.54</td>
<td>&lt;0.0001</td>
<td>1.5</td>
<td>0.81</td>
</tr>
<tr>
<td>Constant</td>
<td>12.4</td>
<td>0.27</td>
<td>0</td>
<td>41.55</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>0.17</td>
<td>0.11</td>
<td>0.14</td>
<td>1.55</td>
<td>0.126</td>
</tr>
<tr>
<td>---------------------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>Supplementation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>period</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 0</td>
<td>-0.05</td>
<td>0.13</td>
<td>-0.03</td>
<td>-5.12</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CRP</td>
<td>-0.97</td>
<td>0.19</td>
<td>-0.47</td>
<td>4.14</td>
<td>0.0005</td>
</tr>
<tr>
<td>Ferritin</td>
<td>0.007</td>
<td>0.001</td>
<td>0.36</td>
<td>3.32</td>
<td>0.0001</td>
</tr>
<tr>
<td>Fat</td>
<td>0.008</td>
<td>0.002</td>
<td>0.32</td>
<td>-3.82</td>
<td>0.002</td>
</tr>
<tr>
<td>Model</td>
<td>6.47</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Constant</td>
<td>-213.7</td>
<td>69.5</td>
<td>0</td>
<td>-3.33</td>
<td>0.0016</td>
</tr>
<tr>
<td>Supplementation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>period</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 0</td>
<td>-0.54</td>
<td>7.06</td>
<td>-0.01</td>
<td>-0.08</td>
<td>0.58</td>
</tr>
<tr>
<td>Ferritin (ng/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>22.95</td>
<td>4.49</td>
<td>0.50</td>
<td>4.60</td>
<td>0.0002</td>
</tr>
<tr>
<td>bPRP</td>
<td>38.76</td>
<td>14.4</td>
<td>0.29</td>
<td>2.69</td>
<td>0.003</td>
</tr>
<tr>
<td>% max iron</td>
<td>-0.70</td>
<td>0.40</td>
<td>-0.19</td>
<td>-1.72</td>
<td>0.0004</td>
</tr>
<tr>
<td>Zinc</td>
<td>-2.24</td>
<td>0.85</td>
<td>-0.32</td>
<td>-2.63</td>
<td>0.022</td>
</tr>
</tbody>
</table>

SE: standard error; CRP: C-reactive protein; bPRP: basic proline rich protein; aPRP: acidic proline rich protein; iAUC: area under the curve; Zn: zinc

Significance: $p < 0.05$
Table 5: Correlations between % max iron absorption and salivary proteins at Week 0 and Week 4 of each supplementation period

<table>
<thead>
<tr>
<th>Dose</th>
<th>Week 0/Week 4</th>
<th>N</th>
<th>bPRP</th>
<th>aPRP</th>
<th>gPRP</th>
<th>Statherin</th>
<th>Cystatin</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R (p)</td>
<td>R (p)</td>
<td>R (p)</td>
<td>R (p)</td>
<td></td>
<td>R (p)</td>
<td>R (p)</td>
<td>R (p)</td>
</tr>
<tr>
<td>Week 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.03 g</td>
<td>0.218</td>
<td>-0.18</td>
<td>-0.131</td>
<td>-0.241</td>
<td>0.290</td>
<td>-0.078</td>
<td>(0.518)</td>
<td>(0.596)</td>
</tr>
<tr>
<td></td>
<td>0.605*</td>
<td>-0.198</td>
<td>0.184</td>
<td>-0.055</td>
<td>-0.007</td>
<td>0.09</td>
<td>(0.049)</td>
<td>(0.56)</td>
</tr>
<tr>
<td>Week 4</td>
<td>0.25</td>
<td>-0.645*</td>
<td>-0.204</td>
<td>-0.245</td>
<td>0.326</td>
<td>0.047</td>
<td>(0.46)</td>
<td>(0.03)</td>
</tr>
<tr>
<td></td>
<td>0.489</td>
<td>0.057</td>
<td>0.111</td>
<td>0.112</td>
<td>0.138</td>
<td>0.158</td>
<td>(0.07)</td>
<td>(0.876)</td>
</tr>
<tr>
<td>Week 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25 g</td>
<td>0.297</td>
<td>0.075</td>
<td>0.391</td>
<td>0.01</td>
<td>0.201</td>
<td>0.46</td>
<td>(0.438)</td>
<td>(0.861)</td>
</tr>
<tr>
<td></td>
<td>0.173</td>
<td>-0.483</td>
<td>-0.595*</td>
<td>0.10</td>
<td>0.114</td>
<td>-0.076</td>
<td>(0.611)</td>
<td>(0.133)</td>
</tr>
<tr>
<td>Week 4</td>
<td>0.366*</td>
<td>-0.20*</td>
<td>-0.23</td>
<td>0.07</td>
<td>0.27*</td>
<td>0.20</td>
<td>(0.003)</td>
<td>(0.028)</td>
</tr>
</tbody>
</table>

basic proline-rich protein: bPRP, acidic proline-rich protein: aPRP, glycosylated proline-rich protein (gPRP); *p < 0.05
Table 6: Mean astringency ratings, and changes from Week 0 to Week 4 of supplementation periods

<table>
<thead>
<tr>
<th>Dose*</th>
<th>0.03 g Week 0</th>
<th>0.03 g Week 4</th>
<th>0.25 g Week 0</th>
<th>0.25 g Week 4</th>
<th>1.5 g Week 0</th>
<th>1.5 g Week 4</th>
<th>Fisher’s exact p Week 0 to 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>1.4</td>
<td>1.1</td>
<td>1.2</td>
<td>1.3</td>
<td>1.2</td>
<td>1*</td>
<td>0.31</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(1.1, 1.6)</td>
<td>(0.9, 1.3)</td>
<td>(0.9, 1.4)</td>
<td>(1.0, 1.5)</td>
<td>(0.9, 1.4)</td>
<td>(0.8, 1.2)</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>2.2</td>
<td>2.3</td>
<td>2</td>
<td>1.9</td>
<td>2.1</td>
<td>1.8*</td>
<td>0.50</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(1.8, 2.5)</td>
<td>(1.9, 2.6)</td>
<td>(1.7, 2.4)</td>
<td>(1.6, 2.3)</td>
<td>(1.7, 2.4)</td>
<td>(1.5, 2.2)</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>3.3</td>
<td>3.4</td>
<td>3.2</td>
<td>3.5</td>
<td>2.4</td>
<td>3.3+</td>
<td>0.126</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(2.8, 3.7)</td>
<td>(2.9, 3.8)</td>
<td>(2.7, 3.6)</td>
<td>(3, 3.9)</td>
<td>(1.9, 2.8)</td>
<td>(2.8, 3.7)</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>4.2</td>
<td>4.4</td>
<td>4.3</td>
<td>4.5</td>
<td>3.3</td>
<td>4.4</td>
<td>0.55</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(3.7, 4.7)</td>
<td>(3.9, 4.8)</td>
<td>(3.8, 4.7)</td>
<td>(4, 5)</td>
<td>(2.9, 3.8)</td>
<td>(3.9, 4.8)</td>
<td></td>
</tr>
</tbody>
</table>

CI: confidence interval

*p < 0.05 Week 4 vs. Week 0. + p < 0.05, 0.03 g vs. 1.5 g supplementation period.

Scale 1 = not astringent, 5 = extremely astringent
Figure Legends:

Figure 1: Enrollment allocation.
Forty-eight potential participants responded to the study call and were screened; the study was conducted on a rolling basis. Twelve participants were initially enrolled, and during study duration, two participants dropped out due to relocation, one due to time commitment in the study, another due to intolerance to blood draws, and one due to supplement intolerance. These participants all dropped out after supplementation period I of the study was completed, and another four participants were recruited on a rolling basis from the initially screened pool of 48.

Figure 2: Supplementation periods (A) and supplementation period activities (B).
The study consisted of three supplementation periods for each participant, a high (1.5 g), medium (0.25 g), or low (0.03 g) condensed tannin supplement was provided for four weeks. Two to three-week washout periods between supplementation periods aimed to stabilize salivary protein and iron biomarkers. Supplementation periods consisted of Week 0 and Week 4 meal challenges, salivary collection, and astringency testing. At midpoint of each supplementation period, there were 3 24- hour dietary recalls (2 weekday, and 1 weekend day) collected from each participant.

Figure 3: Mean adjusted and unadjusted individual level iron absorption at Week 0 and Week 4 of each supplementation period.
Adjusted for hemoglobin, ferritin, C-reactive protein, basic proline-rich proteins, and acidic proline-rich proteins, (red) and unadjusted (black). There were no significant differences ($ps > 0.05$) in iron absorption at any dose of condensed tannin before or after supplementation periods.

Figure 4: Individual level incremental area under the curve for serum iron at Week 0 and Week 4 of each supplementation period.

Mean regression-adjusted for C-reactive protein, basic proline-rich protein, total salivary protein, meat consumption, and rating of highest level of astringency: red; and unadjusted: black. There were no significant differences ($ps > 0.05$) in iron absorption within tannin supplementation periods.
Initial response to recruitment: 48

Originally interviewed and enrolled: 12

Dropped out: Relocation = 2
Time commitment = 1
Side effect = 1
Blood draws = 1

Remaining: 7

Screened and enrolled from initial pool: 4

Total: 11
### Intervention 1
- n = 11
- high, medium, or low dose tannin supplement
- Washout (2-3 weeks)

### Intervention 2
- n = 11
- high, medium, or low dose tannin supplement
- Washout (2-3 weeks)

### Intervention 3
- n = 11
- high, medium, or low dose tannin supplement

---

### Week 0
- Meal challenge:
  - Iron absorption curve
  - Salivary collection
  - Astringency testing

### Midpoint
- 3-24-hour diet recalls (2 weekdays, and 1 weekend day)
- 2-week supplement
- 3 times daily

### Week 4
- Meal challenge:
  - Iron absorption curve
  - Salivary collection
  - Astringency testing

2-week supplement
3 times daily