Non-everted oxygenated rat intestinal segments as a measure of neutral detergent fiber effects on iron absorption

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*Iron absorption in the presence of varying amounts and sizes of dietary fiber was measured. A method using non-everted rat intestinal segments perfused in oxygen was refined. Neutral detergent fiber (NDF), a component of dietary fiber, was extracted from cooked pinto bean (*Phaseolus vulgaris*). The NDF did not affect iron absorption in intestinal segments from iron replete rats. However, 4 and 6 mg of NDF/ml significantly decreased iron absorption in the intestinal segments from anemic rats. NDF with a smaller particle size of 0.125 mm increased iron absorption relative to that absorbed with 0.180 mm particles. Histological examination validated using non-everted intestinal segments perfused with oxygen as a method for studying dietary effects on iron absorption. Segments which are not everted are less prone to damage. Perfusion with oxygen maintained metabolic activity in the tissue during the experiment.*

Keywords: fiber; iron; NDF; *Phaseolus vulgaris;* pinto bean

Introduction

Dietary fiber will form insoluble complexes with iron.[1,2] These complexes may decrease iron bioavailability, possibly leading to iron deficiency in populations with high fiber intakes.[3] Some sources of fiber are reported to decrease iron absorption in humans and in animals, mainly the rat.[4,5] Reinhold et al.[6] reported that neutral detergent fiber (NDF) from wheat and maize decreased iron absorption in rat intestinal segments (cut open lengthwise). NDF decreased iron absorption throughout the jejunum and ileum. This effect was most pronounced when maize fiber was used. Stiles et al.[7] found corn pericarp and cellulose decreased absorption of 59Fe by 15% in the rat. However, other studies have not found significant reduction in iron absorption due to added fiber. Ranhotra et al.[8] found no correlation between fiber and the relative biological value to anemic rats of iron in five types of Iranian breads. Buth and Mehta[9] found that neither citrus pectin nor Phyllum hust (hemicellulose) incorporated into biscuits at 9.17% wt/wt decreased the hemoglobin or hematocrit levels in African green monkeys when fed for 16 months. Other laboratories have also reported conflicting effects of dietary fiber.[10-12]

Anemic rats were shown to be less discriminatory towards the iron available from a diet than were iron replete rats. Fairweather-Tait and Wright[13] reported iron replete rats previously fed with a low iron diet absorbed more iron than those previously fed with a high iron diet. Wolbling et al.[14] found that 30 mg and 100 mg of guar gum and sodium alginate inhibited iron absorption by 60% in iron replete rats. When the same amount of fiber was fed to anemic rats, only a 20% and 30% respective decrease in iron absorption was observed. Becker et al.[15] also observed that a significantly higher concentration of fucoidan was required to obtain the same reduction in iron absorption in anemic versus iron replete rats.

It is likely that conflicting information has arisen from use of everted intestinal segments in prior studies. Eversion stresses the tissue and without perfused oxygen, the tissue may expire, changing the mechanism of iron transport through the tissue. Wide use of iron depletion-repletion techniques to study fiber effects have contributed little to understanding iron

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absorption phenomenon in non-anemic subjects. Our purpose was to evaluate the effect of dietary fiber on iron absorption using both iron replete and anemic rat duodenum-jejunum segments. We have refined a previously published method which does not require eversion and uses perfused oxygen to preserve metabolic activity in the tissue segments. The effects of varying fiber particle size on iron absorption were also explored using this method.

Materials and methods

Two experiments are reported. The first evaluated the effect of fiber concentration on iron absorption by non-everted intestinal segments from iron deficient or iron replete rats. This was accomplished in a 2 × 4 factorial design. Four levels of fiber (0, 2, 4, and 6 mg NDF/ml) and two levels of iron status (i.e., iron deficient and iron replete) were investigated. The second experiment measured the effect of fiber particle size on iron absorption by non-everted intestinal segments from iron replete rats, in a straightforward paired comparison.

Animals and treatments

Weanling male Sprague-Dawley rats were obtained from Harlan Sprague-Dawley (Madison, WI). Upon receipt, animals were housed individually in stainless steel cages with wire mesh floors. Lighting was cycled to provide 12-hour darkness. Feed in aluminum cups and water in rubber-stopped glass bottles were supplied ad libitum. Weight and feed consumption were recorded. Animals were divided into two groups, iron deficient and iron replete. Both groups were fed a standard AIN-76 diet[16] (*Table 1*) with tap water until they reached approximately 200 g wt. The iron deficient group was fed the same diet (*Table 1*) except that added iron was omitted and demineralized water provided. When iron deficient rats reached a hematocrit value of less than 35%, the study began. All diet ingredients were obtained from Teklad Laboratories (Madison, WI). The mineral mix was free of copper, zinc, and iron and was added during preparation of the diet at levels of 5, 12, and 35 mg/ diet, respectively. The mineral sources used were $FeSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, and $ZnSO_4 \cdot 7H_2O$ (Baker Chem. Co).

Table 1 Purified diet formulation used to feed all animals as specified by AIN-76.[16] Iron depleted animals were fed this diet without added $FeSO_4 \cdot 7H_2O$

Ingredient	%
Lactalbumin	20.0
Corn Starch	15.0
Sucrose	50.0
K-Cellulose	5.00
Corn Oil	5.00
AIN Mineral Mix[a]	3.50
$FeSO_4 \cdot 7H_2O$	0.00050
$ZnSO_4 \cdot 7H_2O$	0.00120
$CuSO_4 \cdot H_2O$	0.00350
AIN Vitamin Mix	1.00
Choline Bitrate	0.20

[a] AIN Mineral mix was free of Cu, Fe, and Zn. The minerals were added during mixing of the diet at 5, 12, and 35 mg/kg, respectively.

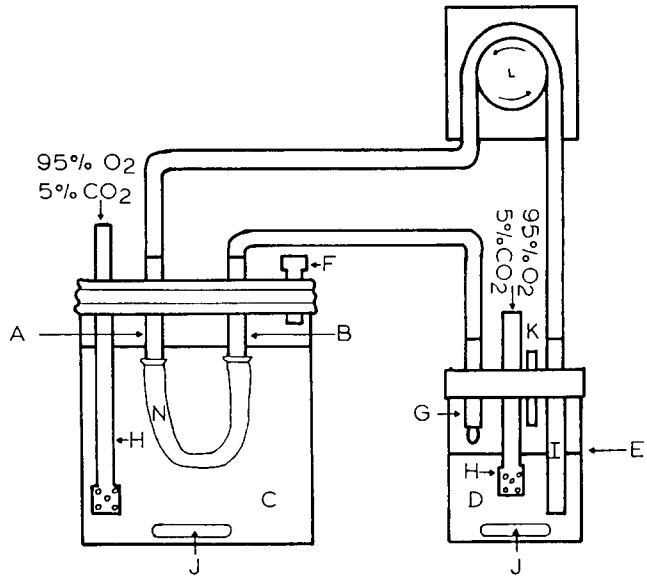

Figure 1 Schematic of a non-everted intestinal perfusion apparatus. A and B are teflon tubes used to cannulate the intestinal segment. C is the serosal fluid reservoir; D is the luminal fluid; E is the reservoir; F and K are portals to withdrawing of serosal and luminal fluid samples; G is the reentry for the luminal fluid; H are the gas dispersion tubes; I is the exit portal for the luminal fluid; J are stirring bars; L is a peristaltic pump; and N is the non-everted intestinal segment. A 95% oxygen gas mixture maintained metabolic activity in the intestinal segment during the experiment, about 1 hour.

Intestinal experiments and apparatus

Non-everted duodenal-jejunum rat intestinal segments were used in all experiments. Five rats were assigned per group so that their group average hemotocrit levels were not different. A method developed by Lyons et al.[17] to study histamine uptake was used here to study uptake of iron from fiber. A major advantage to this method is the use of a 5% CO_2 and 95% O_2 gas mixture which maintains metabolic activity in the intestine for the duration of the experiment. Each rat was anesthetized and killed by decapitation. Ten cm of a non-everted duodenum-jejunum segment was excised and gently rinsed with glucose saline solution (GSS) at 37°C to remove lumen content. The GSS contained 128 mM NaCl, 28 mM D-glucose and 4 mM KCl. Segments were placed in a circulation apparatus as illustrated in *Figure 1*. Segments were attached by cannulation at both ends to teflon tubing (A) and (B) [all capital letters in parentheses refer to *Figure 1*] and tied with surgical thread to prevent losses at the ends of the segments. All were immersed in 70 ml of CMRL 1066 tissue medium (GIBCO, Grand Island, NY) fortified with 1 g/ml insulin and 0.1 g/ml hydrocortisone at pH 7.4. The serosal fluid (C) as well as the luminal fluid (D) were continuously oxygenated by bubbling

with a 95% O_2, 5% CO_2 gas mixture. One end of the cannula (A) was connected to the outlet of a peristaltic pump while the second end (B) was connected to the reservoir (E). Reservoir (E) was connected to the peristaltic pump to form a continuous loop. Reservoir (E) contained luminal fluid (D) which had this composition; 10 ml of CMRL 1066 tissue medium, 0, 20, 40, or 60 mg of NDF, 1 ppm FE and enough 59Fe as $59FeCl_3$ (New England Nuclear, Boston, MA) to give 10,000 cpm. The luminal fluid (D) was circulated at a flow rate of 1.6 ml/min for 60 minutes. A 20 µl aliquot was taken from the serosal fluid (C) and placed into 10 ml Aquasol R™ (New England Nuclear, Boston, MA) and counted in a liquid scintillation spectrometer (Packard Model 3320). Radioactivity in the luminal fluid and serosal fluid was measured at the beginning and end of the experiment. After perfusion, intestinal segments were removed, washed with GSS, and weighed. About 3 mm of each intestinal segment was fixed in 10% neutral buffer (Formalin) containing 0.5% cetyltrimetilammonium bromide for histological examination. The remaining intestine was digested in 3 ml concentrated H_2SO_4 and its radioactivity measured using a Tandem Gamma well detector (Packard Model 3320). Total counts were recorded and these values are reported here as a direct measure of iron absorption.

The second experiment studied the effect of particle size on iron absorption in iron replete rat intestinal segments. For this, the highest level of 6 mg of NDF/ml was used. The rats initially weighed between 75 g and 90 g. The animals were fed the AIN-76 diet shown in *Table 1* until they reached approximately 200 g wt. All rats were killed humanely by anesthetization in methyl ether and by decapitation after fasting for 24 hours. Intestinal segments were removed and treated in the same manner as for experiment one.

Fiber and iron sources

Neutral detergent fiber (NDF) extracted from cooked pinto beans was used in all experiments. NDF was purified using the method of Robertson and Van Soest[18] as later modified by Reinhold and Garcia L.[19] All NDF used in the first experiment was ground by mortar and pestle to pass a 60 coarse mesh screen. For the second experiment, NDF was ground to pass through a 120 fine mesh screen (0.125 mm particle size) and a 60 but not 80 mesh screen (0.18 mm particle size). A 0.01 M $FeSO_4 \cdot 7H_2O$ in 0.1 N HCl solution, to which 20 µl of $59FeCl_3$ (New England Nuclear, specific activity 11.31 C/g) was added, served as the stock iron solution.

Biochemical and analytical measures

Hemoglobin and hematocrit levels were measured in blood obtained by cutting the small tip of the tail. Hemoglobin was measured using the cyanomethemoglobin method.[20] Drabkin's solution as well as the hemoglobin standard were obtained from Sigma. Hematocrit levels were determined using heparinized micro-capillary tubes.

After tissues were fixed in buffered Formalin solution, they were frozen in O.C.T. embedding medium (Tissue-Tek II) and sectioned onto 20 micron slides with a Lypshaw-Microtome Model 1500. The sections were stained with Gill-3 hematoxylin (Lerner Lab., Sandford, CO) for 1.5 minutes, de-stained with demineralized water, and examined by a trained pathologist. Data were analyzed using Minitab statistical software by unpaired *t*-tests, one-way analysis of variance, and least significant difference (LSD).[21]

Results and discussion

Anemic versus iron replete rats

Table 2 is the hemoglobin concentration, hematocrit levels, animal weights, and iron absorbed as total counts per minute of 59Fe per gram of intestine for the anemic and iron replete treatments. Iron replete rats were fed for a longer time than the anemic group as shown by significantly higher body weights. A few rats in the iron replete group developed an eye infection during the growing period and were removed from the study. Only healthy rats are included in these results. The hemoglobin concentration in the anemic group (4.63 to 4.91 g/dl, *Table 2*) was below the value specified by Pla and Fritz[22] for an anemic rat. Therefore, our animals were considered in a well-defined anemic state. Iron replete rats had twice the hemoglobin concentration and three times the hematocrit level than did those in the anemic group. As desired, the mean weight, hematocrit, and hemoglobin levels did not differ within each treatment (i.e., replete or anemic treatments in *Table 2*) but were, of course, different between treatments ($P < 0.01$).

Total counts per minute (CPM) of 59Fe per gram of intestinal tissue are reported as a direct measure of iron absorption. *Table 2* also shows the CPM for both groups at different concentrations of fiber. One-way analysis of variance shows significant differences between mean CPM values. A subsequent range test (LSD) identified differences within each group and between treatments, indicated by letters a, b, and c in *Table 2*.

NDF did not significantly affect iron absorption, independent of fiber concentration in iron replete animals. A trend toward lower absorption as the level of fiber increased exists. Group three (4 mg NDF/ml) was eliminated because the intestinal segments were found damaged upon histological examination. Within the anemic group, fiber significantly decreased ($P < 0.05$) iron absorption at levels of 4 and 6 mg NDF/ml but not at 2 mg NDF/ml. A trend towards increased iron absorption was observed between the 4 and 6 mg NDF/ml groups. These data are similar to those reported by Becker et al.[15] who found increased iron absorption in the presence of 0.6 mg fiber per ml when using iron replete rats. This increase was also observed at 25 mg/ml when using anemic rats. There was

Table 2 Iron availability as a function of fiber concentration and iron status of the rat. Biochemical indices of iron status are shown for iron replete and anemic rats from which intestinal segments were taken for each test

NDF fiber mg/ml	Animals: Iron status	Animals: n	Weight g	Hematocrit %	Hemoglobin g/dl	Absorption of 59Fe, CPM/g
0	Replete	5	227.1 ± 4.1[a]	44.8 ± 1.7[a]	12.50 ± 0.54[a]	15.0 ± 5.2[a]
2.0	Replete	4	234.4 ± 13.2[a]	44.0 ± 1.4[a]	13.02 ± 0.47[a]	15.2 ± 4.3[a]
6.0	Replete	5	227.8 ± 18.3[a]	44.2 ± 2.2[a]	13.33 ± 0.64[a]	13.0 ± 3.1[a]
0	Anemic	4	155.0 ± 8.7[b]	22.9 ± 1.6[b]	4.91 ± 0.47[b]	23.1 ± 3.0[c]
2.0	Anemic	4	155.9 ± 16.0[b]	24.6 ± 1.8[b]	4.88 ± 0.58[b]	23.0 ± 2.1[c]
4.0	Anemic	4	153.0 ± 8.5[b]	23.7 ± 4.3[b]	4.63 ± 0.33[b]	16.0 ± 2.0[a,b]
6.0	Anemic	5	151.7 ± 8.8[b]	23.8 ± 1.2[b]	4.89 ± 0.46[b]	18.1 ± 2.2[b]

[a,b,c] Means in the same column [vertical] followed by the same letter are not significantly different ($P < 0.05$) by one-way analysis of variance and least significant difference (LSD) test.

Table 3 The effect of fiber particle size on iron absorption using iron replete rats. Hemoglobin concentrations, hematocrit levels, and weights are shown for rats from which duodenal-jejunal segments were removed with corresponding counts per minute (CPM) per g tissue segment. Data are means ±1 SD

Particle Size, mm	Number of Rats	Mean Weight g	Hematocrit %	Hemoglobin g/dl	CPM per g tissue
0.125 Fine	4	182.9 ± 11.9	41.7 ± 1.9	12.67 ± 0.64	21.12 ± 1.15[a]
0.18 Coarse	6	180.3 ± 15.7	41.5 ± 1.5	13.52 ± 0.77	17.39 ± 1.56[a]

[a] Difference significant between particle sizes at $P < 0.01$.

no significant difference in the total amount of iron absorbed by anemic and iron replete rats. However, the anemic group required higher concentrations of fiber to obtain equivalent decreases in iron absorption as did the iron replete rats. Wolbling et al.[14] and Becker et al.[15] reported results similar to ours. Anemic rats have a greater ability to absorb iron, possibly associated with the presence of free binding sites on the mucosa.[23] This suggests that fiber may decrease iron absorption by entrapment within the polymer, with the iron on the surface available for absorption. This suggestion is supported by the higher absorption observed in iron replete rats with finer fiber particle sizes.

We have found NDF from cooked pinto beans to bind relatively large amounts of iron (i.e., 5.85 μg iron per mg NDF).[24] At high iron concentrations, a large proportion of the iron may be weakly associated with fiber but bound with sufficient strength to prevent its precipitation. The increased ability of the anemic mucosa to absorb iron and the weaker association of iron with fiber at higher iron concentrations may account for the higher iron absorption when 2 mg of fiber but not 6 mg of fiber were used.

Particle size

An effect of fiber particle size on iron absorption is shown in *Table 3*. Test groups did not differ in body weight, hematocrit levels, or hemoglobin concentrations at time of sacrifice as designed. Fiber with the smaller 0.125 mm particle size allowed greater iron absorption than the group with larger 0.18 mm particle size ($P < 0.01$). These results contrast those of Caprez and Fairweather-Tait[10] who reported no effect of bran particle size on iron availability to the rat. More iron absorbed with the small particle size implies that fiber may act as an expeditor of iron absorption in iron replete rats. We have found previously that fiber binds more iron as particle size decreases.[24] The practical implications of fiber particle size remain to be explored.

Conclusion

Perfusion of non-everted rat intestinal segments may be a useful test for effects of dietary components on iron absorption. Non-everted intestinal segments have not previously been used to study effects of fiber on iron absorption. Non-everted intestines require less handling than everted segments. Thus, they suffer less stress and more reproducible information may be obtained through their use. Histological examination of our tissues showed 75% of the intestinal segments were in good condition at the end of the study, and this provided evidence of sustained metabolic activity. Data from damaged or expired segments were not used.

This investigation has shown that fiber may either decrease or increase iron absorption depending upon fiber particle size, anemic or replete iron status, and the fiber concentration. Fiber appears to inhibit iron absorption by direct entrapment of iron within the fi-

ber polymer. The use of smaller particle size fiber may increase food iron absorption and this deserves further study.

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