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Iron Bioavailability of Rats Fed Liver, Lentil, Spinach and their Mixtures

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Abstract: To study the effects of dietary iron source (basal diet-FeSO₄.7H₂O, liver, lentil, spinach, liver + lentil, liver+spinach and lentil+spinach) on iron bioavailability, fifty-six Albino Sprague Dawley derived male 21 days old rats were fed on iron-deficient diet (7.8 mg Fe kg⁻¹ diet) and the mentioned seven iron containing diets (40 mg Fe kg⁻¹ diet) for 10 days. Rats fed liver diet showed higher iron apparent absorption (52.1%), hemoglobin (Hb) gain (0.94 g/100 mL), Hb-iron gain (1.2 mg), Hb-regeneration efficiency (HRE%) (50.8%), relative efficiency of HRE% (106.5%), packed cell volume gain (2.22%) and mean corpuscular hemoglobin concentration (0.64 g dL⁻¹). Liver resulted in an increase in these parameters when mixed with lentil and spinach diets. However, rats fed iron free diet showed the higher dry matter absorption.

Key words: Iron, hemoglobin, rats, liver, lentil, spinach

INTRODUCTION

Iron deficiency anemia remain the most common nutritional deficiency in the world (Pinero *et al.*, 2000; Stoltzfus *et al.*, 2004). Over the last 60 years, a great number of studies were executed to study the iron metabolism. These studies were concerned with blood, medulla and endogenous organs: liver and spleen (Bothwell *et al.*, 1958; Bothwell *et al.*, 1979; Dallman *et al.*, 1980; Bjorn-Rasmussen, 1983). There are three disorder states can be resulted from iron deficiency: iron deficiency (the absence of iron deposits); ferrodeficiency erythropoiesis (insufficient supply of iron to the medulla) and ferropenic anemia (reduction the hemoglobin value below tolerable limit) (Bothwell *et al.*, 1979; Cook and Finch, 1979; Rodrigues-Matas *et al.*, 1998). Knowledge about the iron absorption and factors affecting it has increased considerably since the extrinsic tag was introduced to label dietary iron in meals (Cook *et al.*, 1972; Hallberg and Bjorn-Rasmussen, 1972). Dietary iron exists in two forms, they are heme iron in animal sources and non heme iron in plant sources and in some animal foods, as are nonheme enzymes and ferritin. Several factors affect the intestinal absorption of iron, especially non heme iron. Some food contain absorption- enhancing

factors such as ascorbic acid from plant sources (Siegenberg *et al.*, 1991) and the so called meat factor which contained in animal meats like liver (Cook and Reddy, 2001). The form of iron in food may affect its absorption. Heme iron is much better absorbed than non heme iron. Food with a high phytate content have low iron bioavailability, but whether the phytate is the cause is not clear. Oxalates can inhibit absorption.

This study was designed to evaluate the effects of heme iron, non heme iron, phytate and oxalate on iron bioavailability.

MATERIALS AND METHODS

Animals: This study was executed at Balgaa University, 2008. Albino Sprague Dawley derived male 21 days old rats were used. At the beginning, the rats were given free access to distilled water and fed a non purified diet (rodent diet) for the first 3 days. From the acclimated rats, 56 rats were divided into eight groups (7 rats each) in such a manner that both hemoglobin (Hb) concentration and body weight were approximately the same for each group. Animals were housed individually in suspended stainless steel cages having wires mesh bottoms and fronts in room maintained at 23-25 and 12 h

light: dark cycle (light on between 8:00 am to 8:00 pm daily). Weighted foods and redistilled water were provided *ad libitum*. The animals and food scrap were weighted and faces were collected daily. All cages, water bottles and mixer bowls that were used for diet preparations, were rinsed with 13.7 mmol L⁻¹ EDTA and washed by double distilled water to minimize mineral contamination.

Diets: Table 1 shows the composition of eight diets. They contained casein-vit. free (BDH Chem. Ltd. Co., England), vitamin and mineral mixtures as indicated later. Corn starch, corn oil, sucrose, liver, lentil, spinach and cellulose purchased from local market. The ingredients for each diet were blended in an electric stainless steel mixer to a homogeneous, moistened with redistilled water, pasted, pelted (4-6 g), dried by cabinet dryer at 55°C and stored in a freezer. Iron concentrations of the diets were determined after mixing and iron in all iron containing seven diets ranged from 39.1-41.1 mg kg⁻¹ diets, while the iron-deficient diet contained about 7.8 mg kg⁻¹ diet. To prepare the fresh liver, lentil and spinach, the liver were sliced to 1-2 cm, blanched by redistilled water in glass beaker for 10 min and spread at stainless steel dishes; lentil and spinach were cleaned by redistilled water and all three samples were dried by cabinet dryer at 55°C. All dried samples of liver, lentil and spinach were milled by electrical blender and stored in freezer to the next procedures.

Chemical analysis: Twelfth hours after the last feeding, the rats were weighted and anesthetized with chloroform and blood samples were collected directly from the heart with a heparinized syringe. Parts of collected blood samples were transferred to a heparinized tube and used for determination of packed cell volume (PCV) and Hb concentration, the remainder was centrifuged at 4°C for

20 min at 1400 x g and the serum was separated and stored at -18°C to the next analysis. The rats liver and spleen, were excised weighed and stored at -18°C for further analysis. The proximate composition of dried liver, lentil and spinach and of diets was determined using the following (AOAC, 1984) methods: moisture (14.004), fat (14.018), fiber (14.020) and nitrogen (14.026). The conversion factor of nitrogen to brotinen was 5.7 for lentil and spinach and 6.2 for liver. Total mineral content was obtained by calcination of 5 g of the dried liver, lentil and spinach and diets and feces of rats in an muffle furnace at 550°C until complete calcination was achieved, then the ash was solved in 6 N HCl and filtered. The aliquot was received by volumetric flask and the volume was completed to 100 mL by demineralized distilled water and used to analysis. Total iron of dried liver, lentil and spinach and of diets was determined according to Schrickler *et al.* (1982). Non heme iron concentration in liver and in blood Hb were determined according to Miller *et al.* (1994) and King *et al.* (1990), respectively. Total iron in blood Hb was determined according to Trinder (1956). Heme- iron was calculated by the different (total iron-minus non heme iron). Oxalic acid and phytic acid were determined according to Trevaskis and Trenerry (1996) and Wheeler and Ferrel (1971), respectively. Blood Hb was determined according to Trinder (1956). PCV was determined by centrifugation of blood into heparinized micro capillary tubes. Apparent iron absorption (AIA%) was calculated by the formula:

$$AIA(\%) = \frac{\text{mg iron intake} - \text{mg fecal iron}}{\text{mg iron intake}} \times 100$$

Dry matter absorption (DMA%) was calculated by the formula:

$$DMA(\%) = \frac{\text{g diet intake} - \text{g diet in feces}}{\text{g diet intake}} \times 100$$

Table 1: Composition of diets (g kg⁻¹)

Ingredients	Diets name							
	Iron free	Basal	Liver	Lentil	Spinach	Liver+Lentil	Liver+Spinach	Lentil+Spinach
Casein	140	140.00	9	32	115	21	62	44
Corn starch	503	503.00	455	170	453	313	454	312
Corn oil	150	150.00	132	145	146	139	139	146
Cellulose	50	50.00	50	34	43	42	47	39
Sucrose	90	90.00	90	90	90	90	90	90
Vit.-mixture*	15	15.00	15	15	15	15	15	15
Mineral-mixture*	20	20.00	20	20	20	20	20	20
Phosphate	18	18.00	11	12	16	14	14	14
Calcium	14	14.00	14	13	10	12	13	12
Iron	-	0.42	-	-	-	-	-	-
Dried liver	-	-	204	-	-	101	101	-
Dried lentil	-	-	-	469	-	233	-	233
Dried spinach	-	-	-	-	92	-	45	54

Vitamin mixture contained (g kg⁻¹): vitamin A (retinyl acetate), 6.88; vitamin D3 (calciferol), 0.125; alphatochopherol, 4.0; thiaminhydrochloride, 1.0; riboflavin, 4.0; pyridoxine hydrochloride, 3.0; niacin, 10.0; folic acid, 0.5; calcium pantothenate, 5.0; vitamin B12, 0.005; choline hydrochloride, 75.0; ascorbic acid, 45.0; biotin, 0.02; menadion, 125.0 and dextrose to make 1 kg mixture. Mineral mixture contained (g kg⁻¹): FeSO₄.7H₂O, 3.5; KCl, ZnSO₄, 0.2; MnSO₄.H₂O, 2.0; CuSO₄, 4.0; KI, 0.08; MgCO₃, 28 and dextrose to make 1 kg mixture

Hb- iron gain was calculated as the difference between Hb-iron at the end of repletion period (10 days) and that at the start of repletion. For calculating the initial and final Hb-iron, blood was assumed to be 67 g kg⁻¹ body wt. and Hb was assumed to contain 3.35 mg iron g⁻¹ (Cartland and Koch, 1928). The next formula was used to calculate Hb-iron:

$$\text{Hb-iron (mg)} = \text{body wt. (BW)} \times (6.7 \text{ mL blood } 100 \text{ g}^{-1} \text{ BW}) \times (\text{g Hb } 100 \text{ mL}^{-1} \text{ blood}) \times (3.35 \text{ mg Fe/1.0 g Hb})$$

The hemoglobin regeneration efficiency (HRE) was calculated for each rat as the percentage of iron consumed that was retained in circulating Hb (Forbes *et al.*, 1989) and the next formula was used:

$$\text{HRE\%} = \frac{\text{mg final Hb-iron} - \text{mg initial Hb-iron}}{\text{mg iron consumed}} \times 100$$

Relative efficiency (RE) % of HRE of used diet was calculated by the formula:

$$\text{RE\%} = \frac{\text{HRE\% of any diet}}{\text{HRE\% of basal diet}} \times 100$$

Mean corpuscular hemoglobin concentration (MCHC) 100 mL = Hb/PCV ×100 (Cartland and Koch, 1928).

Statistical analysis: The data reported are the Means+Standard error (SE). Statistical differences between the study groups were evaluated by independent t-tests method. The differences were considered significant at p< 0.05 (Steel and Torrie, 1982).

RESULTS AND DISCUSSION

The primary analysis show that the iron content (mg/100 g) of dried liver, lentil and spinach was 20.1, 8.7 and 44.6, respectively. On the base of iron content of these dried foods, the experimental diets were formulated as shown in Table 1.

Table 2 show the analysis of the eight diets of the study. From Table 2, the average percents of protein, fat and fiber for these diets were about 13.7, 14.7 and 5, respectively. The average contents (mg kg⁻¹ diet) of ascorbic acid and (g kg⁻¹ diet) of phosphor and calcium were 0.87, 6 and 2.5, respectively. Iron content (mg kg⁻¹ diet) ranged from 7.8 in iron- free diet to about 40 as an average of seven iron-containing diets. Oxalic acid (mg kg⁻¹ diet) only found in spinach diet (30.1) and its mixture diets: liver + spinach diet (16.8) and lentil + spinach diet (17.4). Phytic acid (g kg⁻¹ diet) only found in lentil diet (0.84) and its mixture diets: liver + lentil diet (0.38) and lentil +spinach diet (0.40).

Data of Table 3 show that the average percent of AIA and DMA of rats fed these diets were 47.7 and 91.5,

Table 2: Chemical analysis of diets

Diets	Protein (%)	Fat (%)	Fiber (%)	Ascorbic (mg kg ⁻¹)	Oxalic (mg kg ⁻¹)	Phytic (g kg ⁻¹)	Iron (mg kg ⁻¹)	Phosphorus (g kg ⁻¹)	Calcium (g kg ⁻¹)
Iron free	13.6	14.7	5.0	0.88	-	-	7.8	5.43	2.82
Basal	13.7	14.8	5.0	0.87	-	-	41.0	5.32	2.76
Liver	13.8	14.9	4.9	0.86	-	-	40.0	6.31	2.30
Lentil	13.5	14.6	5.1	0.91	-	0.84	39.1	6.25	2.65
Spinach	13.9	14.5	5.2	0.84	30.1	-	39.6	6.13	2.41
Liver+Lentil	13.7	14.7	4.9	0.87	-	0.38	40.0	6.28	2.40
Liver+Spinach	13.6	14.6	5.0	0.84	16.8	-	40.1	6.20	2.37
Lentil+Spinach	13.6	14.5	5.1	0.86	17.4	0.40	39.3	6.21	2.47

Table 3: Nutritional status and growth response of rats fed liver, lentil, spinach and their mixtures

Parameters	Diets							
	Iron free	Basal	Liver	Lentil	Spinach	Liver+Lentil	Liver+Spinach	Lentil+Spinach
Body wt. (g)								
Initial	53.400±1.82	58.300±1.20	59.40±1.60	57.30±0.80	60.200±1.53	50.300±1.71	54.700±0.93	63.500±1.40
Gain	3.200±0.25 ^a	37.400±2.30 ^{ab}	42.50±2.80 ^a	33.60±1.80	30.400±2.30	38.900±1.51 ^{ab}	37.300±1.73 ^{ab}	30.700±1.73 ^b
Liver								
Weight (g)	2.320±0.04 ^a	4.870±0.08 ^{ab}	5.02±0.07 ^a	4.610±0.12 ^{bc}	4.230±0.09 ^{cd}	4.070±0.12	4.150±0.14 ^d	4.310±0.05 ^{cd}
Iron content (mg)	0.184±0.02 ^a	0.391±0.01 ^{ab}	0.41±0.03 ^a	0.351±0.02 ^a	0.335±0.01 ^{cd}	0.327±0.01 ^d	0.330±0.03 ^d	0.338±0.03 ^{cd}
Spleen								
Weight (g)	0.150±0.04 ^a	0.410±0.02 ^{ab}	0.44±0.03 ^a	0.40±0.02 ^{ab}	0.360±0.01 ^b	0.410±0.01 ^{ab}	0.430±0.01 ^a	0.340±0.03 ^b
Iron content (mg)	0.120±0.01 ^a	0.310±0.03 ^{ab}	0.32±0.03 ^a	0.24±0.02 ^c	0.170±0.02	0.270±0.03 ^b	0.250±0.04 ^{bc}	0.210±0.01 ^d
Food intake								
Weight (g)	109.600±2.50 ^f	115.100±2.30 ^f	112.30±3.1 ^{ab}	107.20±1.80 ^b	90.200±2.1 ^d	108.000±2.60 ^f	96.400±3.20 ^f	92.000±2.70 ^{cd}
Iron content (mg)	0.860±0.05 ^e	4.720±0.25 ^a	4.53±0.27 ^{ab}	4.20±0.31 ^{bc}	3.570±0.28 ^{cd}	4.400±0.34 ^b	3.900±0.30 ^c	3.800±0.32 ^c
Feces								
Weight (g)	6.210±0.37 ^a	8.300±0.41 ^a	9.85±0.44 ^{ab}	10.15±0.53 ^a	8.060±0.61 ^{cd}	9.700±0.71 ^{ab}	8.580±0.64 ^{bc}	8.980±0.55 ^b
Iron content (mg)	0.460±0.04 ^f	2.770±0.08 ^a	2.17±0.10 ^c	2.28±0.11 ^b	1.870±0.12 ^e	2.190±0.09 ^{bc}	1.920±0.07	2.010±0.06 ^d
AIA%*	46.500±0.10 ^{bc}	41.400±0.13 ^e	52.10±0.08 ^a	45.70±0.09 ^{bc}	47.600±0.14 ^b	50.200±0.16 ^{ab}	50.800±0.07 ^{ab}	47.200±0.05 ^b
DMA%*	94.300±0.24 ^a	92.800±0.08 ^{ab}	91.20±0.13 ^{abc}	90.50±0.17 ^{bc}	91.100±0.21 ^{abc}	91.000±0.18 ^{bc}	91.100±0.09 ^{bc}	90.300±0.10 ^{bc}

*AIA: Apparent iron absorption; DMA: Dry matter absorption. All values are expressed as Mean±SEM. Values in a row not sharing a common superscript are significantly different

Table 4: Hemoglobin regeneration efficiency (HRE), relative efficiency (RE), packed cell volume (PCV) and mean corpuscular hemoglobin concentration (MCHC) of rats fed liver, lentil, spinach and their mixtures

Parameters	Diets							
	Iron free	Basal	Liver	Lentil	Spinach	Liver+Lentil	Liver+Spinach	Lentil+Spinach
Absorbed iron (mg)								
Total	0.40±0.01 ^f	1.95±0.05 ^c	2.36±0.08 ^a	1.92±0.10 ^c	1.70±0.03 ^e	2.21±0.12 ^b	1.98±0.14 ^e	1.79±0.06 ^d
Heme	-	-	0.76±0.03 ^a	-	-	0.42±0.04 ^b	0.38±0.03 ^{bc}	-
Non-heme	0.40±0.01 ^e	1.95±0.05 ^a	1.60±0.05 ^d	1.92±0.10 ^a	1.70±0.03 ^e	1.79±0.06 ^b	1.60±0.07 ^d	1.79±0.06 ^b
Hemoglobin (g dL⁻¹)								
Initial	10.92±0.15	10.76±0.13	10.35±0.21	9.87±0.25	10.18±0.23	10.05±0.18	9.73±0.19	10.42±0.22
Gain	0.66±0.01 ^d	0.61±0.03 ^e	0.94±0.02 ^a	0.78±0.01 ^{bc}	0.51±0.02	0.82±0.03 ^b	0.67±0.04 ^d	0.62±0.04 ^e
Hemoglobin-iron (mg)								
Total iron	1.39±0.03 ^d	2.34±0.08 ^b	2.58±0.07 ^a	2.18±0.05 ^c	2.17±0.08 ^e	2.18±0.06 ^e	2.15±0.05 ^e	2.33±0.04 ^b
Initial	1.31±0.06	1.41±0.05	1.38±0.04	1.27±0.03	1.38±0.05	1.13±0.07	1.19±0.08	1.49±0.04
Gain	0.08±0.01 ^f	0.93±0.02 ^c	1.20±0.02 ^a	0.91±0.01 ^{cd}	0.79±0.04 ^e	1.05±0.03 ^b	0.96±0.05 ^e	0.84±0.04 ^d
Heme-iron								
Initial	1.01±0.01	1.10±0.02	1.05±0.03	0.98±0.04	1.06±0.03	0.90±0.01	0.92±0.02	1.15±0.03
Gain	0.05±0.00	0.73±0.03	1.02±0.02	0.71±0.01	0.62±0.01	0.83±0.02	0.75±0.04	0.66±0.03
Non-heme-iron								
Initial	0.30±0.01	0.31±0.01	0.23±0.02	0.29±0.03	0.32±0.01	0.23±0.01	0.27±0.02	0.34±0.03
Gain	0.03±0.01 ^e	0.20±0.02 ^a	0.18±0.03 ^{ab}	0.20±0.01 ^a	0.17±0.02 ^b	0.22±0.02 ^a	0.21±0.01 ^a	0.18±0.01 ^{ab}
HRE%	20.00±0.80 ^d	47.70±1.30 ^{bc}	50.80±1.50 ^a	47.40±1.60 ^{bc}	46.50±1.40 ^e	47.50±1.70 ^{bc}	48.50±1.80 ^b	46.90±2.10 ^c
RE%	41.90±1.2 ^d	100.00±1.60 ^{bc}	106.50±1.40 ^a	99.40±1.60 ^{bc}	97.50±1.80 ^e	99.60±1.90 ^{bc}	101.80±2.20 ^b	98.30±2.3 ^c
PCV%								
Initial	33.40±0.50	34.90±0.70	31.80±0.40	38.70±0.60	39.60±0.50	36.80±0.80	35.90±1.00	39.20±1.10
Gain	0.30±0.01 ^f	1.30±0.03 ^d	2.22±0.02 ^a	1.99±0.04 ^b	1.23±0.05 ^{de}	2.15±0.04 ^{ab}	1.57±0.05 ^e	1.55±0.03 ^e
MCHC (g dL⁻¹)								
Initial	32.70±0.60	30.80±0.70	32.60±0.80	25.60±0.70	25.70±1.00	27.30±0.80	27.10±1.10	26.60±0.50
Gain	0.51±0.01 ^e	0.57±0.04 ^b	0.64±0.03 ^a	0.43±0.01 ^e	0.48±0.01 ^d	0.61±0.02 ^a	0.52±0.02 ^c	0.49±0.03 ^d

All values are expressed as Mean±SEM. Values in a row not sharing a common superscript are significantly different

respectively; the liver diet resulted in the higher AIA percent (52.1); the rats fed iron-free diet had the higher DMA percent (94.3). Lentil and spinach in as alone diets or in mixture diets resulted in reduction in these two parameters. From the 1940s, where the biosynthetic tagging of individual foods introduced by Moore and Dubach (1951), two fundamental observations were showed: the iron from animal sources is better assimilated than that from plant sources and its absorption from the latter is significantly enhanced by ascorbic acid (Hallberg *et al.*, 1989; Fleming *et al.*, 2002). Iron in the intestinal tract is absorbed as the reduced iron and this imply that iron must be in reduced and soluble form during the process of iron absorption (Apte and Venkatachalam, 1965; Kuhn *et al.*, 1968). Thus the ability of dietary component to enhance bioavailability of iron could be attributed to its ability to maintain iron in the reduced form and/or the ability to complex with iron keeping it in solution and available for absorption. The high AIA showed in rats fed liver diet may due to its cysteine content that inhibit formation of insoluble complex of iron-fiber, which reduce iron absorbability (Zhag *et al.*, 1989; Zimmermann *et al.*, 2005) and to change some casein in basal diet by liver protein which may be resulted in showing the meat factor that increase iron absorption. However liver heme iron may increase absorption of non heme iron (Layerisse and Roch, 1968; Hallberg *et al.*, 1989; Rodriguez *et al.*, 2007) and this role showed when liver mixed with lentil and spinach which

only contained non heme iron. The reduction effect of iron absorption which showed in rats fed lentil and spinach diets may due to the phytate content in the former and oxalate in the latter (Navert *et al.*, 1985; Hallberg *et al.*, 1987; Hallberg *et al.*, 1989). Other dietary constituents may also affect the utilization of iron, dietary fiber, tannins and perhaps other unknown factors (Farah *et al.*, 1984; Dintzis *et al.*, 1985; Hallberg *et al.*, 1987). Results of iron absorption were in agreements with findings of Zhang *et al.* (1989). However, the higher percent of DMA which showed in rats fed iron-free diet may due to high need of these rats to iron.

Table 3 show that the body weight gain (g) and weight (g) of liver and spleen were respectively ranged from 3.2, 2.32 and 0.15 for rats fed iron-free diet to about 36, 4.5 and 0.4 as an average for rats fed seven iron-containing diets. Feeding liver to the rats resulted in the higher body weight gain (42.5 g), liver weight (5.02) and spleen weight (0.44) than those in rats fed other diets. However, this higher weight of liver and spleen of rat group fed liver diet may be parallel ed the higher final weight (about 102 g) of this group. Iron content (mg) of liver and spleen as shown in Table 3 ranged from 0.18 and 0.12, respectively, in rats fed iron-free diet to about 0.35 and 0, 25, respectively, as an average of rat group; fed seven iron-containing diets. Liver diet resulted in the higher content of iron in rat liver (0.41) and in rat spleen (0.44), whereas lentil and spinach as alone diets or in mixture diets resulted in reduction in these contents and

may due to the higher absorbed iron (2.78 mg) of rats fed liver diet than that of rats fed lentil diet (2.11 mg) and spinach diet (1.83 mg).

From Table 4, the gains of Hb (g dL⁻¹), Hb-iron (mg), PVC (%) and calculated MCHC% were respectively ranged from 0.06, 0.08, -0.34 and 0.51 in rats fed iron-free diet to about 0.71, 0.95, 1.72 and 0.53 as an average of rat groups fed seven-iron containing diets. Liver diet resulted in the higher gains of Hb (0.94), Hb-iron (1.2), PCV (2.22) and of MCHC (0.64) than those with the basal and other diets and caused raising effect in these parameters when mixed with lentil and spinach. Increased gains of these parameters in rats fed liver diet may due to change some casein in basal diet by liver protein and to the higher absorbed iron compared with those of rats fed lentil and spinach diets which may be contained reduction factors (Gad *et al.*, 1982; Lee and Hendricks, 1995).

To evaluate the effect of the study diets on the bioavailability of iron, HRE and RE were calculated and this procedure was deemed to be a good predictor (Forbes *et al.*, 1989). The calculated HRE and RE value reflects the ability of rats to utilize and retain dietary iron. The effects of experimental diets on these parameters after the 10 days feeding period are shown in Table 4. Table 4 shows that the liver diet resulted in the higher HRE (50.8%) and RE (106.9%) of basal diet than the other diets.

In conclusion, the results of this study showed that the iron deficiency is tightly associated with body and endogenous development. The liver diets may enhance the bioavailability of iron from plant sources and may be the better source for iron deficiency treatment.

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