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Dietary iron supplements and *Moringa oleifera* leaves influence the liver hepcidin messenger RNA expression and biochemical indices of iron status in rats

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ABSTRACT

In this study, the effects of iron depletion and repletion on biochemical and molecular indices of iron status were investigated in growing male Wistar rats. We hypothesized that iron from Moringa leaves could overcome the effects of iron deficiency and modulate the expression of iron-responsive genes better than conventional iron supplements. Iron deficiency was induced by feeding rats an iron-deficient diet for 10 weeks, whereas control rats were maintained on an iron-sufficient diet (35.0-mg Fe/kg diet). After the depletion period, animals were repleted with different source of iron, in combination with ascorbic acid. Iron deficiency caused a significant ($P < .05$) decrease in serum iron and ferritin levels by 57% and 40%, respectively, as compared with nondepleted control animals. Significant changes in the expression (0.5- to100-fold) of liver hepcidin (HAMP), transferrin, transferrin receptor-2, hemochromatosis type 2, ferroportin 1, ceruloplasmin, and ferritin-H were recorded in iron-depleted and iron-repleted rats, as compared with nondepleted rats ($P < .05$). Dietary iron from Moringa leaf was found to be superior compared with ferric citrate in overcoming the effects of iron deficiency in rats. These results suggest that changes in the relative expression of liver hepcidin messenger RNA can be used as a sensitive molecular marker for iron deficiency.

1. Introduction

By contributing in a wide variety of metabolic processes, including oxygen sensing, iron is essential to all organisms [1]. Iron deficiency is one of the principal risk factors for disability and death, affecting an estimated 2 billion people worldwide. According to estimates of the World Health Organization, in developing countries, 48% of children between the ages of 5 and 14 years and 52% of pregnant women, including India, are anemic [2]. Continuous consumption of plant-based diets is the basic cause of low-iron bioavailability, due to low absorption of non-haem iron (<10% bioavailable) as compared with haem iron (15%-35% bioavailable), which is predominantly present in animal tissues [3,4]. Cereals and legumes

Abbreviations: AA, ascorbic acid; FPN1, ferroportin 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HAMP, hepcidin antimicrobial peptide; HFE2, hemochromatosis type 2; ID, iron deficient; MO, *Moringa oleifera*; TFR-2, transferrin receptor-2; UBC, ubiquitin-conjugating enzyme 18.

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also contain high amounts of phytic acid (inositol phosphates), iron-binding phenolic compounds, and calcium, which further inhibit the absorption of iron in animals [3]. Iron requirements increase 3-fold during pregnancy because of the expansion of maternal red cell mass and fetal-placental growth [5]. Over the past decade, major advances have been made in understanding the molecular mechanism of iron homeostasis, and this has led to the identification of key proteins, including hepcidin, ferroportin, ferritin, transferrin, and ceruloplasmin (CP). The coordinated regulation of these genes at transcriptional and translational levels controls iron absorption and transportation during iron-deficient (ID) and replete conditions [6].

Dietary modification, diversification, and iron fortification of foods can control iron deficiency in populations. Biofortification using plant breeding and genetic engineering is also a new approach. Although dietary modification and diversification is the most appropriate and sustainable tactic, modification of dietary preferences is difficult, and foods that deliver highly bioavailable iron are expensive. Water-soluble and highly bioavailable iron salts react with other food components and cause off-flavors, color changes, and fat oxidation, which limit the scope of iron fortification in foods.

A sustainable food-based approach that uses dietary sources of iron, in adequate amounts, can be effective in controlling iron deficiency and other commonly associated nutritional deficiencies. In view of the significant content of iron and other nutritionally important phytoconstituents in leaves of Moringa [7–9], it could be an affordable and sustainable source of iron to make fortified foods that address iron deficiency problems, at least in places where it is commonly grown. Moringa leaves contain a high amount of iron (17.5 mg/100 g Dry weight), compared with other common leafy vegetables such as green amaranthus (*Amaranthus viridis*), bathua (*Chenopodium album*), and kenaf (*Hibiscus cannabinus*) leaves (R.K. Saini, N.P. Shetty, and P. Giridhar, unpublished data). Use of the fresh leaves, flowers, and tender pods (fruits) of this plant as a vegetable is confined to African and Asian countries, including India [10,11]. In India, the value of tender pods and seed oil of *Moringa oleifera* for medicinal use is well known [12–14]. The potent antistress, antioxidant, and scavenging potential of the leaves and fruits of *M oleifera* have been established by *in vitro* and in *vivo* studies [15,16]; however, there is no information on the bioavailability of iron from fresh or dehydrated Moringa leaves.

Predicting iron bioavailability is difficult because several factors such as, species, matrix, and absorption modifiers may influence iron absorption [17]. Moringa leaves also contain high amounts of ascorbic acid (AA) and carotenoids (220 and 80 mg/100 g Fresh weight (FW), respectively) [18], which are known enhancers of iron absorption [17]. Ascorbic acid reduces the ferric form of iron (Fe^{+3}) into ferrous (Fe^{+2}) in duodenum, whereas divalent metal ion transporter 1 (DMT1) transports only ferrous iron [19].

The present investigation was carried out with the hypothesis that dietary iron supplements from *M oleifera* leaves could influence the molecular and biochemical indices of iron status more than conventional iron supplements. Our study design allowed us to investigate the specific effects of the different levels of dietary iron on the content of serum iron, ferritin, and transferrin and modulation of the levels of messenger RNA (mRNA) encoding proteins involved in iron bioavailability, intestinal absorption, and liver iron metabolism. In order to test this hypothesis, a well-established iron depletion and repletion approach was used [20]. We first depleted the iron by maintaining the animals on an ID diet. After the depletion period, animals were repleted with different sources of iron, and the effect on serum lipids, iron, ferritin, and transferrin levels were measured. The expression pattern of liver iron-responsive genes was also recorded in iron-depleted and iron-repleted rats.

2. Methods and materials

2.1. Materials

Fresh leaves were collected from 3-year-old *M. oleifera* (cv. PKM-1) plants that were grown in the Central Food Technological Research Institute's orchard during the month of December 2012 (average temperature was 23-25°C). Recommended cultural practices were followed to raise the plants on the field [21]. The fresh leaves from the top third petiole were collected in the early morning, mixed thoroughly, and dried using a cabinet tray dryer (Armstrong Smith, Mumbai, India) with a capacity of 40 trays of 400 × 800 mm at 50°C [22]. Dehydrated leaves were powdered and stored at –80°C in amber-color air-tight containers until use. Content of total iron in the dehydrated leaves was determined using Atomic Absorption Flame Emission Spectrophotometer Shimadzu AA-6710F Shimadzu, Kyoto, Japan after ashing in a muffle furnace [23].

Refined Groundnut oil (Fortune; Adani Wilmar Limited, Gujarat, India), corn starch, and cane sugar were procured from Loyal World supermarket Mysore. Minerals, vitamins, cellulose, choline chloride, and L-cystine were purchased from Himedia Laboratories (Mumbai, India). Casein was purchased from the Nimesh Corporation (Mumbai, India). Clinical enzyme kits for the estimation of iron, ferritin, transferrin, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides, total cholesterol, glucose, albumin, and total protein were purchased from Agappe Diagnostics (Kerala, India).

2.2. Rats and diets

Male Wistar rats (OUTB-Wistar, IND-cft [2c]) weighing 60 ± 5 g (approximately 24 days old) were used in this study. The experimental protocol was approved by the institute's Animal Ethical Committee. Animals were grouped according to the outline given in Fig. 1, divided into groups of 4, and then housed in metabolic cages, under a 12-hour light/dark cycle with 25 ± 2°C and 40% to 60% relative humidity. The diet plan, duration of treatment, and detailed iron depletion and repletion schedule are given in Fig. 1. Diet ingredient compositions (AIN-93M) shown in Table 1 were formulated based on previous work [24]. In brief, a total of 58 rats were divided into 4 groups that consisted of 12 and 46 rats of equal mean weights. For 10 weeks, the smaller group was fed the control diet (no depletion), which contained 35 mg iron/kg of diet, whereas the larger group was fed with the same diet without iron. At the end of the 10 weeks, 6 iron-depleted and 6 control rats were

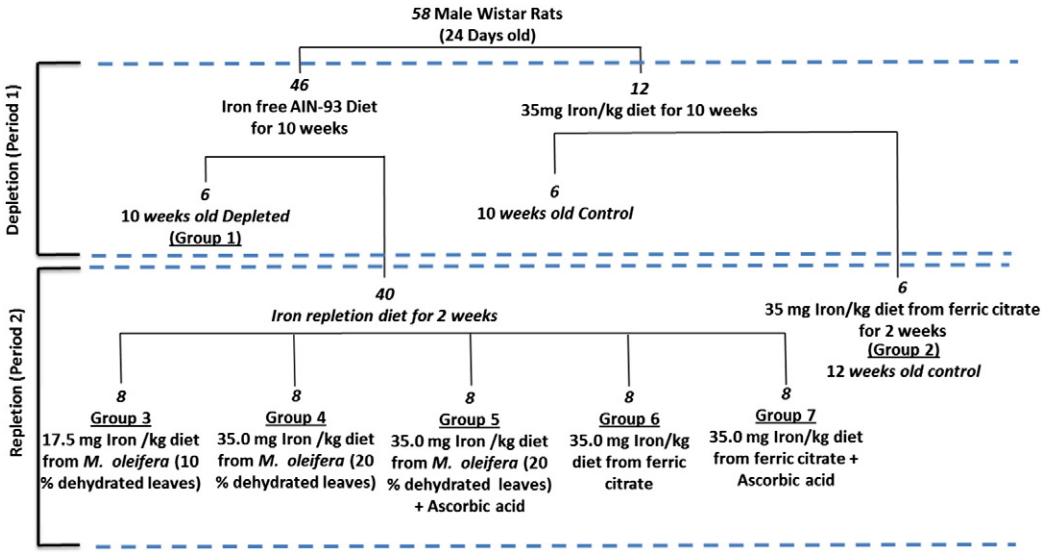

Fig. 1 – Diagram is the flowchart of the protocol for iron depletion and repletion rats. Italicized text represents the age of the various groups when blood, serum, and liver were removed for analysis. Italicized numbers indicate the number of animals in each group.

fasted overnight and euthanized under ether anesthesia. Blood was drawn by cardiac puncture, and serum was separated by centrifugation at 4°C. Livers were dissected and then rinsed with Diethyl pyrocarbonate-treated water. Approximately 1 g of liver sample was frozen in liquid nitrogen in 15 mL RNase-free falcon tubes and then immediately stored at –80°C for RNA extraction. Serum clinical enzymes and metabolites, such as iron, ferritin, transferrin, LDL, HDL, total cholesterol, triglycerides, glucose, albumin, and total protein, were analyzed by using Agape diagnostic kits, (Pattimattom, Kerala, India) as per the manufacturer's guidelines.

The remaining 6 control rats continued to receive the control diet (no depletion) containing 35 mg iron/kg of diet for the next 4 weeks (12-week-old, control). The remaining 40 rats (iron depleted) were divided into 5 groups of equal mean weights. The groups were randomly assigned to the iron-repleted diet for 4 weeks and had different sources of iron from ferric citrate and Moringa leaves, in combination with AA. In the Moringa leaf–based diet, 10% and 20% dehydrated Moringa leaves were incorporated, at the expense of corn starch, to supply 17.5- and 35.0-mg Fe per kg diet. To study the effect of AA supplementation on iron absorption, diets were prepared in the presence and absence of AA at 2:1 molar ratio (AA/Fe) [25]. At the end of the repletion period, all rats were anesthetized, blood was collected, and livers were excised and processed as explained above.

Table 1 – Ingredient composition of the iron-depleted and iron-repleted diets

Ingredients	ID diet	Control (no depletion) diet	Depleted + 10% MO diet	Depleted + 20% MO diet	Depleted + 20% MO + AA diet	Depleted + 35.0-mg Fe diet	Depleted + 35.0-mg Fe + AA diet
Corn starch	500.0	500.0	400.0	300.0	300.0	500.0	500.0
Casein (85% protein)	200.0	200.0	200.0	200.0	200.0	200.0	200.0
Sucrose	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Groundnut oil	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Fiber (cellulose)	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Mineral mix[a]	35.0	35.0	35.0	35.0	35.0	35.0	35.0
Vitamin mix (AIN-93-VX)	10.0	10.0	10.0	10.0	10.0	10.0	10.0
L-Cystine	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Choline bitartrate (41.1% choline)	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Moringa dry leaves	0	0	100	200	200	0	0
AA	0	0	0	0	0.217	0	0.217
Ferric citrate	0	0.1953	0	0	0	0.1953	0.1953

All values are in g/kg diet. MO, *M oleifera*.
[a] AIN-93G-MX; deficient in iron.

Table 2 – List of primers/genes used to assess iron metabolism in the rat

S / No.	Primer/gene name	Forward primer	Reverse primer	Tm	PCR product length	Gene ID (NCBI)
1	Ferroportin 1 (*FPN1*)	TCTATATGACGGTCCTGGGC	GCCAGTCCTGAGAACAGACC	60	187	AF394785.3
2	Ceruloplasmin (*CP*)	CTTCCAATACAAGCACAGGG	CTGTAGCTCTGAGACGATGC	60	195	NM_001270961.1
3	Hepcidin antimicrobial peptide (*HAMP*)	AAGATGGCACTAAGCACTCG	GCATTTACAGCAGAAGAGGC	60	219	NM_053469.1
4	Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	AGAACATCATCCCTGCATCC	AGTCACAGGAGACAACCTGG	60	246	NM_017008.4
5	Ferritin-H (*FH*)	CTTTGCAACTTCGTCGCTCC	GTCCTGGTGGTAGTTCTGGC	60	106	NM_012848.2
6	Hemochromatosis type 2 (*HFE2*)	GCCTACTTCCAATCCTGCGT	GGTCAAGAAGACTCGGGCAT	60	102	NM_001012080.1
7	Transferrin (*TF*)	GATGAGTGGAGCGTCACGAG	CTGGCCTGCTATGTAGGCAT	60	138	D38380.1
8	Transferrin receptor-2 (*TFR-2*)	GGACCCTGACCTTTCCCATC	GACCACTGTTGCACTTTCCG	60	137	BC166451.1

NCBI, National Center for Biotechnology Information.

2.3. *RNA extraction and real-time quantitative polymerase chain reaction analysis*

Total RNA extraction was carried out using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) from 50-mg frozen liver, as per the manufacturer's guidelines. RNA concentration and purity were determined by spectrophotometry, using Nano Drop 1000, NanoDrop Technologies. Inc., Wilmington, DE, USA. After quantification, RNA quality and integrity was analyzed in a 1.5% (wt/vol) agarose formamide gel. Complementary DNA (cDNA) was synthesized in 40 μL reaction, using 2 μg of total RNA, 0.5 mM dNTP mix, 5 μM random hexamer, 5 μM oligo dT primers, 40 units of RNase inhibitor, and 400 units of M-MLV reverse transcriptase (Sigma Aldrich, Bangalore, India). Complementary DNA was diluted in 160 μL of water to achieve 5-fold dilution and then used as the template for real-time quantitative polymerase chain reaction (qPCR). Quantitative reverse transcriptase PCR was performed in a total volume of 10 μL, including 2 μL of diluted cDNA, 0.5 μM for each primer, and 5 μL of 2× SsoFast EvaGreen Supermix (Bio-Rad Inc, Hercules, CA, USA) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Inc). The qPCR procedure included a preliminary step of 95°C for 1 minute; followed by 40 cycles of 95°C for 10 seconds, and 60°C for 20 seconds. Melt curve was performed from 65.0°C to 95.0°C, with increments of 0.5° every 5 seconds. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize the gene expression. The GAPDH content of rat liver was not impacted by dietary minerals [26,27]. Relative gene expression was calculated according to a $2^{-\Delta\Delta CT}$ method [28]. Quantitative PCR was performed in triplicates, and the fold change in each target gene was compared with the nondepleted control (12-week-old) that was set to 1. Primer sequences, Tm values, PCR product length, and National Center for Biotechnology Information accession numbers of the genes used in the study are given in Table 2.

2.4. *Statistical analyses*

Statistical analysis was performed using the SPSS statistics 17.0 (SPSS Inc, Chicago, IL, USA). Data were analyzed by 1-way analysis of variance, at 95% confidence level ($P < .05$), and same results were presented as means ± SD. Homogeneity of variance test was also performed to obtain the "homogeneous subsets" for the means. Correlation studies were also conducted among serum ferritin, transferrin, and iron concentration in order to understand the direct relationships between different indices of iron status. A power analysis was performed online (http://openepi.com) to estimate the number of rats needed to obtain 80% power at a confidence level of 95%. Eight animals would be required to obtain 8% differences in serum iron content among treatment groups. Similarly, 6 animals would be required to obtain 10% differences among treatment and control groups.

Table 3 – Influence of dietary Moringa dehydrated leaves on rat weight, serum iron, ferritin and transferrin content

Measurements	Treatments							
	Control	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
Weight (g)	271.0 ± 6.1^{b}	271.3 ± 7.2^{b}	298.8 ± 10.1^{a}	296.3 ± 15.6^{a}	308.8 ± 19.1^{a}	309.4 ± 20.8^{a}	296.3 ± 20.4^{a}	290.6 ± 26.6^{a}
Iron (μg/dL)	140.4 ± 17.2^{c}	61.5 ± 15.3^{e}	146.5 ± 22.6^{c}	184.3 ± 12.6^{a}	173.9 ± 8.9^{b}	127.4 ± 15.4^{cd}	127 ± 13.7^{cd}	122.8 ± 7.6^{d}
Ferritin (ng/dL)	396.3 ± 12.8^{ab}	237.3 ± 16.8^{c}	418.0 ± 30.0^{a}	415.0 ± 44.6^{a}	417.2 ± 47.1^{a}	346.0 ± 31.0^{b}	224.2 ± 11.9^{c}	257.9 ± 5.6^{c}
Transferrin (ng/dL)	301.1 ± 17.4^{a}	317.6 ± 23.7^{a}	293.3 ± 7.5^{a}	212.7 ± 35.5^{b}	197.3 ± 22.3^{b}	197.6 ± 26.8^{b}	193.5 ± 33.5^{b}	153.4 ± 18.8^{c}

Values are means ± SD of 6 (control) and 8 (treated) animals. Control, nondepleted (10 week); group 1, depleted (10 weeks); group 2, nondepleted (12 week); group 3, depleted + 17.5-mg Fe/kg diet from *M Oleifera* leaves (MO); group 4, depleted + 35.0-mg Fe/kg diet from MO; group 5, depleted + 35.0-mg Fe/kg diet from MO + AA; group 6, depleted + 35.0-mg Fe/kg diet from ferric citrate (FC); group 7, depleted + 35.0-mg Fe/kg diet from FC + AA. Values with different superscript letters indicate statistically significant differences between the means ($P < .05$).

3. Results

3.1. Body weight

Rats fed either of the ID diets (group 1) or control diet (10-week nondepleted control) grew in the same way during the depletion period, as evidenced by nonsignificant final body weights of 271.3 and 271.0 g, respectively (Table 3). During the repletion period, the animals fed with different diets did not show significant differences in body weight. Rats fed the control diet (group 2), 10% Moringa leaf (group 3), 20% Moringa leaf (group 4), 20% Moringa leaf with AA (group 5), 35.0 mg Fe from ferric citrate (group 6), and 35.0 mg Fe from ferric citrate with AA (group 7) did not show any significant difference ($P < .05$) in final body weights (290.6-298.8 g).

3.2. Serum profile

Serum iron concentrations (Table 3) showed a complex pattern in rats fed with different diets. Serum iron level decreased to 61.5 μg/dL in rats fed the Fe-depleted diet for 10 weeks compared with rats fed the nondepleted diet (140.4 μg/dL). The iron content was further increased in rats fed the nondepleted diet for 12 weeks (146.5 μg/dL). During the Fe repletion period (4 weeks), final Serum Fe content was highest (184.3 μg/dL) in rats fed the 10% Moringa leaf diet, followed by the 20% Moringa leaf diet. The addition of AA in a 2:1 molar ratio (AA/Fe) did not show any improvement in serum iron and ferritin content (Table 3). In a correlation study among serum ferritin and iron concentration, concentration of serum ferritin was found concordant with iron ($R^2 = 0.60$). Maximum concentration of serum ferritin was recorded in nondepleted rats (12 weeks) and rats fed the 10% and 20% Moringa leaf diets, which were 418.0, 415.0, and 417.2 ng/dL, respectively. A significantly higher amount of total cholesterol (39.7 mg/dL) was recorded in iron-depleted rats, compared with nondepleted and repleted rats (Table 4). In general, the content of serum triglycerides and total cholesterol was higher in rats repleted with Moringa leaf compared with those receiving the ferric citrate–based diet. Content of serum glucose was higher in the 10-week nondepleted (120.0 mg/dL) control, 12-week nondepleted (119.7 mg/dL) control, and 10-week depleted rats (119.5 mg/dL), compared with the other repleted groups (96.1-112.9 mg/dL). Albumin content was highest in the 12-week nondepleted control rats (2.03 mg/dL) and nonsignificant among all other groups. Likewise, total protein content was similar and nonsignificant among all treated and control groups.

3.3. Expression of genes relevant to iron metabolism

The relative expression pattern of genes relevant to iron metabolism is given in Fig. 2. All of the studied genes showed significantly lower expression in iron-depleted rats compared with the nondepleted control. The relative expression of liver hepcidin antimicrobial peptides (Hamp) was recorded 100-fold less in iron-depleted rats compared with the nondepleted control (1.0). Similarly, a significantly lower expression ($P < .05$) of hepcidin gene was recorded in rats repleted with 20% Moringa leaves (0.47), 20% Moringa leaves with AA (0.43), and ferric citrate with AA, compared with the nondepleted control (1.0).

Expression of hemochromatosis type 2 (HFE2) was also noted to be significantly lower (0.35-0.48) in the iron-depleted and all repleted groups, when compared with nondepleted control. Expression of transferrin receptor 2 (TFR-2) was lowest in the rats repleted with ferric citrate (0.4), as compared with the nondepleted control (1.0); whereas the expression of CP was recorded at a maximum (2.4) in the same treatment group. In contrast to other genes, expression of CP was higher in all the repleted groups (1.2-2.4), as compared with nondepleted control (1.0).

4. Discussion

Dietary iron supplements from Moringa leaves were found to be more beneficial in overcoming the effects of iron deficiency, when compared with ferric citrate. Among the studied genes, changes in relative expression of liver hepcidin mRNA was most significant, thereby confirming our research hypothesis.

In the present study, *M oleifera* was selected because the aim was to establish that this readily available and iron-rich,

Table 4 – Influence of dietary Moringa dehydrated leaves on serum lipids, glucose, and protein content

Measurements	Treatments							
	Control	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
Triglycerides (mg/dL)	116.1 ± 16.2^{b}	128.9 ± 20.4^{a}	112.2 ± 23.5^{b}	138.9 ± 27.5^{a}	128.1 ± 8.3^{a}	117.8 ± 30.4^{b}	110.0 ± 18.4^{b}	116.1 ± 32.2^{b}
Cholesterol								
LDL (mg/dL)	5.4 ± 1.5^{c}	5.4 ± 1.35^{c}	9.6 ± 1.96^{b}	11.2 ± 2.06^{ab}	12.6 ± 1.87^{a}	11.0 ± 1.81^{ab}	10.7 ± 3.12^{ab}	9.3 ± 2.02^{b}
HDL (mg/dL)	22.7 ± 1.8^{ab}	22.6 ± 1.94^{ab}	21.2 ± 2.54^{b}	21.1 ± 3.78^{b}	21.9 ± 2.31^{ab}	26.5 ± 5.77^{b}	20.9 ± 3.44^{b}	20.5 ± 4.48^{a}
Total (mg/dL)	33.2 ± 7.6^{b}	39.7 ± 8.5^{a}	32.2 ± 7.7^{b}	30.8 ± 4.3^{b}	30.4 ± 3.7^{bc}	29.5 ± 6.2^{bc}	21.6 ± 4.3^{d}	23.1 ± 6.3^{cd}
Glucose (mg/dL)	120.0 ± 5.8^{a}	119.5 ± 6.1^{a}	119.7 ± 5.6^{a}	108.4 ± 8.9^{bc}	112.9 ± 10.2^{b}	106.7 ± 10.6^{bc}	110.0 ± 11.9^{bc}	96.1 ± 11.8^{c}
Total protein (g/dL)	6.7 ± 0.8^{a}	6.9 ± 1.1^{a}	6.7 ± 0.2^{a}	6.9 ± 1.0^{a}	6.7 ± 0.6^{a}	6.6 ± 0.4^{a}	6.9 ± 0.3^{a}	6.5 ± 0.4^{a}
Albumin (g/dL)	1.74 ± 0.3^{ab}	1.67 ± 0.09^{ab}	2.03 ± 0.17^{a}	1.78 ± 0.24^{ab}	1.61 ± 0.27^{b}	1.70 ± 0.41^{ab}	1.50 ± 0.23^{b}	1.49 ± 0.38^{b}

Values are means ± S.D of 6 (control) and 8 (treated) animals. Control, nondepleted (10 week); group 1, depleted (10 weeks); group 2, nondepleted (12 week); group 3, depleted + 17.5-mg Fe/kg diet from *M Oleifera* leaves (MO); group 4, depleted + 35.0-mg Fe/kg diet from MO; group 5, depleted + 35.0-mg Fe/kg diet from MO + AA; group 6, depleted + 35.0-mg Fe/kg diet from ferric citrate (FC); group 7, depleted + 35.0-mg Fe/kg diet from FC + AA. Values with different superscript letters indicate statistically significant differences between the means ($P < .05$).

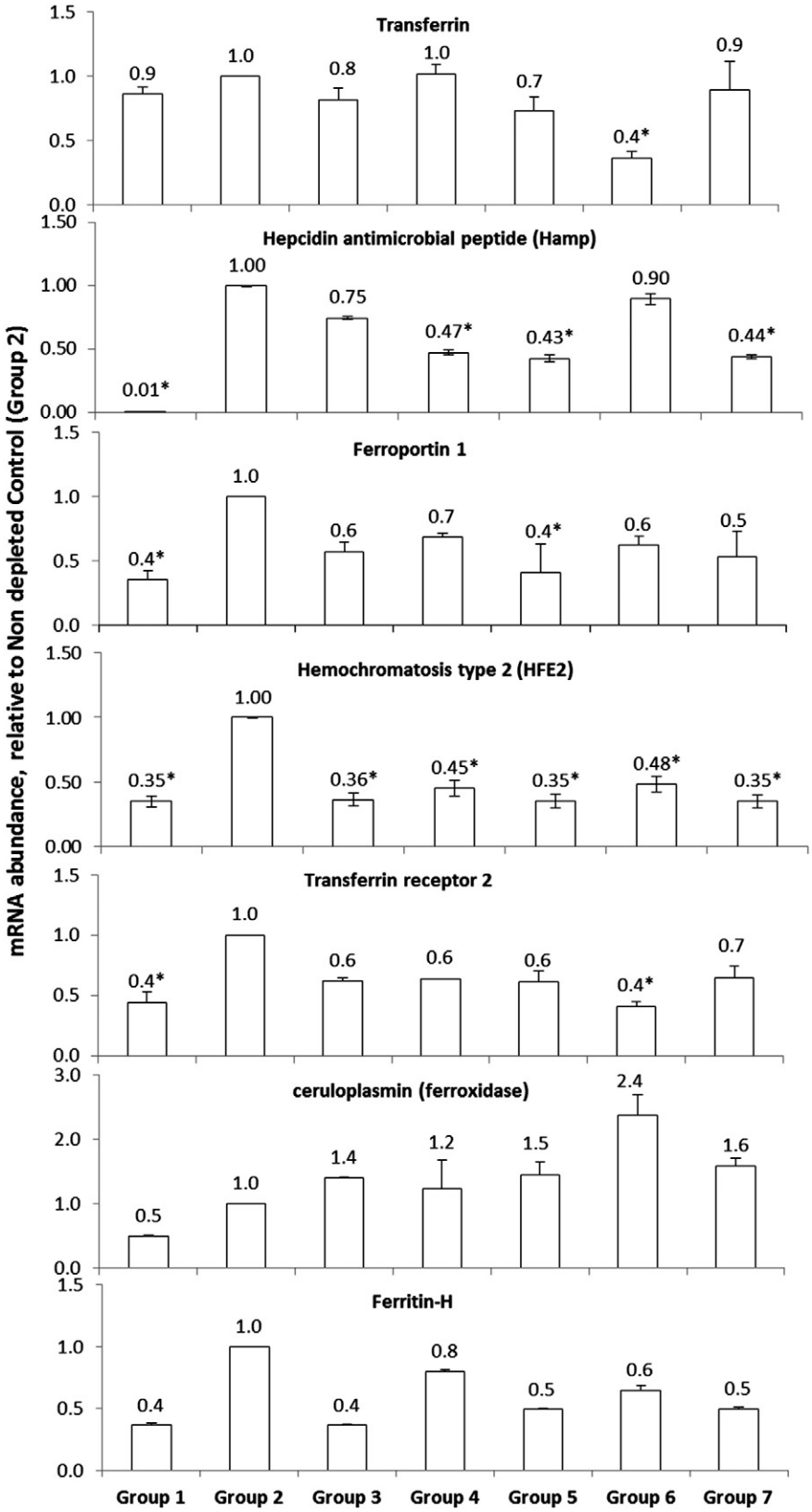

Fig. 2 – Effect of ID and repletion diet on the expression of liver iron transporter and metabolite genes, determined by reverse transcriptase qPCR. Relative transcript abundances of each gene were normalized to housekeeping genes (*GAPDH*). Values are means ± SD. Group 1, depleted (10 weeks); group 2, control (nondepleted, 12 weeks); group 3, depleted + 17.5 mg Fe/kg diet from *M.oleifera* leaves (MO); group 4, depleted + 35.0 mg Fe/kg diet from MO; group 5, depleted + 35.0 mg Fe/kg diet from MO + AA; group 6, depleted + 35.0 mg Fe/kg diet from ferric citrate (FC); group 7, depleted + 35.0 mg Fe/kg diet from FC + AA. *Significant ($P < .05$) compared with nondepleted control (group 2).

but underutilized, leafy vegetable is a source of dietary iron that could aid in the prevention of iron deficiency. In a survey of underutilized leafy vegetables in India, *M oleifera* was noted as the richest in iron content when compared with green amaranthus (*A viridis*), bathua (*C album*), and kenaf (*H cannabinus*) leaves (R.K. Saini, N.P. Shetty, and P. Giridhar, unpublished data). In addition, Moringa leaves have been established as potent antioxidants because of the presence of polyphenolic compounds. Hence, consumption of a diet supplemented with Moringa leaves could protect against oxidative stress [29]. Ferric citrate was used as the source of iron in the control diet because citrate form is well used and less likely to oxidize the food matrix [30]. The diets that differed in the source of dietary iron, ferric citrate, and Moringa leaf, with and without AA supplements, were well accepted by the rats. Consumption of an ID diet for 10 weeks caused significant decreases in serum iron and ferritin content by 57% and 40%, respectively, compared with nondepleted controls (10 weeks). Researchers have used different durations of depletion periods to induce iron deficiency in animals [31,32]. Hence, to determine the appropriate depletion period for iron deficiency, during each week of the depletion period, a small amount of blood (0.5 mL) was drawn (phlebotomy) from retro orbital plexus, and serum iron, ferritin, transferrin, and hemoglobin content was determined. Only 25% and 40% reduction in serum iron content was observed at the end of 4 and 8 weeks of the depletion period, respectively (data not shown). Thus, the animals were provided an ID diet for 10 weeks in order to obtain a serum iron deficiency more than 50%, compared with the nondepleted control. Phlebotomy is also beneficial in inducing iron deficiency in experimental animals [33]. Serum iron and ferritin content were observed as more responsive to the iron depletion diet, compared with hemoglobin and transferrin content (data not shown). Significant decreases in body weights of ID anemic rats were recorded in previous studies [31,32]. However, in the present study, at the end of the 10-week depletion period, significant differences in body weight were not observed among iron-depleted and control rats. It is possible that, in the current study, we obtained the iron deficiency without the anemia. Therefore, reduction of serum iron and ferritin in iron-depleted rats was likely to be caused by factors related to reduced body iron storage, but unrelated to hemoglobin. Studies have also reported the hypocholesterolemic activity of Moringa leaves in high-fat and normal-diet fed rats [34,35]; however, we did not use the high-fat diet in the present study. Therefore, consistent effects on cholesterol and triglycerides levels were not observed in the present investigation.

In rats, the iron from Moringa leaf compared with ferric citrate revealed higher serum iron, ferritin, and transferrin concentration at the end of 4 weeks of with the repletion diet. A low value of serum ferritin is the most appropriate index of iron-induced deficiency [36], and it is directly proportional to the iron store in the body; 1 μg/L serum ferritin is equal to 8 to 10 mg of body iron and 120 μg of storage iron per kg bodyweight [37].

Hepcidin expression was found to be decreased by 100-fold in ID rats (group 1), compared with the nondepleted control rats (group 2), in the present study. This demonstrates the severity of iron deficiency. During repletion of iron with the ferric citrate–based diet (group 6), its expression was restored to near normal (0.9); whereas in the treatment group, serum iron and ferritin levels were not concordant with this expression pattern. Hepcidin expression is a function of iron absorption, and significant changes in its expression can be seen without significant changes in mean cell volume and hemoglobin content [38]. In contrast to this, expression of ferroportin 1 (FPN1) was recorded 40-fold higher than hepcidin, in ID rats (group 1).

The liver plays a crucial role in iron metabolism and expression because almost all the genes are related to iron transportation, metabolism, and homeostasis. Hepcidin antimicrobial peptide, TFR-2, HFE2, FPN1, and CP genes are expressed predominantly in the liver [39]. Heredity mutations in TFR-2, HFE2, or hemojuvelin (HJV) cause severe hemochromatosis (iron overload) due to down-regulation of hepcidin, thus resulting in continuous absorption of iron from the intestine. Hepcidin, an antimicrobial peptide, plays a central role in iron absorption by the intestinal epithelial cells (enterocytes). The expression of hepcidin mRNA increases with iron overloads and prevents further iron absorption by lysosomal degradation of FPN in enterocytes [33]. Therefore, hepcidin mRNA expression correlates inversely with the activity of duodenal iron transporters (FPN1) and iron absorption [38].

Transferrin and TFR-2 also play critical roles in iron homeostasis. In contrast to hepcidin, expression of transferrin and TFR-2 is up-regulated in iron deficiency conditions [40]. However, in ID conditions, we did not observe any significant changes in transferrin expression, whereas significantly lowered expression of TFR-2 was recorded in iron-depleted rats (group 1) when compared with control rats (group 2). Ferritin is a universal intracellular protein consisting of light (L) and heavy (H) subunits that stores and releases iron in a controlled way. Translation of the ferritin gene is up-regulated in iron overload conditions for higher iron sequestration, which prevents iron-mediated oxidative damage. In the present study, concentration of ferritin protein in serum is in agreement with the expression profile of liver mRNA, except in rats that were fed the 10% Moringa leaf diet (group 3).

Ceruloplasmin, a copper containing glycoprotein, plays an important role in the reduction of absorbed ferric iron (Fe^{+3}) into ferrous (Fe^{+2}) through its ferroxidase activity and allows its (Fe^{+2}) incorporation into transferrin and uptake by peripheral tissue. Activity of CP is increased in ID conditions to stimulate iron uptake [41]. In all the treated groups, we noticed a significantly higher abundance of CP mRNA during repletion, as compared with the nondepleted control (1.0). Surprisingly, all of the other genes that were studied showed significantly lower or equal mRNA abundance during depletion and repletion, compared with nondepleted controls. Hemochromatosis type 2 regulates iron absorption by interaction with Fe-transferrin and TFR. In iron overload, these interactions enhance the hepcidin transcription, which prevents intestinal iron absorption through lysosomal degradation of FPN1. Relative changes in the expression profile of liver HFE-2, under ID and balanced conditions, have not been previously recorded. In the present study, a significantly low expression (<0.5-fold) of HFE2 was recorded in all the repleted

and ID rats, compared with the control rats. In the experiments with duodenal HFE expression, no significant difference was observed between the rats fed the ID and balanced diets [42].

It is known that iron absorption from the intestines correlates inversely with the iron status of the body. In the present study, most of the genes showed significantly lower or equal mRNA abundance during iron depletion and repletion. This suggests that down-regulation of iron absorption inhibitory genes, such as hepcidin, ferritin, and HFE-2, plays a key role in enhanced absorption of iron in ID conditions. Enhanced expression of liver TFR-2, TF, and CP genes may not be strictly required in iron deficiency.

Our study has some limitations. First, we were unable to completely eliminate the iron in the ID diet because different minerals used in the formulation of the diet also contributed traces of iron. Second, although a significant reduction in serum ferritin and transferrin was recorded in ID rats, we were unable to observe iron deficiency anemia in the rats. We recorded the mRNA expression of iron-responsive genes in the liver; however, expression of TFR and ferritin proteins is controlled at a posttranscriptional level that is facilitated by the interaction between iron regulatory proteins and iron-responsive elements. Thus, studies focusing on the expression of these proteins may provide a greater understanding of their regulation under iron-depleted and iron-repleted conditions.

In conclusion, dietary iron supplements from Moringa leaves were found to be more beneficial compared with ferric citrate in overcoming the effects of iron deficiency. Changes in the expression (0.5- to 100-fold) of liver hepcidin, transferrin, TFR-2, HFE2, FPN1, CP, and ferritin-H were recorded in iron-depleted and iron-repleted rats, as compared with nondepleted rats. Supplementation of AA in a 2:1 molar ratio (Fe/AA) was not found to be effective in modulating the expression of iron metabolism-related genes and iron absorption. This study suggests that changes in relative expression of liver hepcidin mRNA can be used as a sensitive molecular marker for iron deficiency. The results also suggest that *M oleifera* leaves are a rich source of highly bioavailable iron that can have significant implications in malnutrition programs to alleviate iron deficiency. International nongovernmental organizations, such as Trees for Life and Educational Concerns for Hunger Organization, have vigorously supported Moringa leaves as "natural nutrition for the tropics." India, the world's largest producer of Moringa, is well positioned to provide this effective and low-cost food for the malnourished population.

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