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ORIGINAL CONTRIBUTION

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Does ascorbic acid supplementation affect iron bioavailability in rats fed micronized dispersible ferric pyrophosphate fortified fruit juice?

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Abstract *Background and aims* Food iron (Fe) fortification is an adequate approach for preventing Fe-deficiency anemia. Poorly water-soluble Fe compounds have good sensory attributes but low bioavailability. The reduction of the particle size of Fe fortificants and the addition of ascorbic acid might increase the bioavailability of low-soluble compounds. The present work aims to compare the Fe absorption and bioavailability of micronized dispersible ferric pyrophosphate (MDFP) (poorly soluble) to ferrous sufate (FS) (highly soluble) added to a fruit juice in presence or absence of ascorbic acid (AA) by using the hemoglobin repletion assay in rats. *Methods* After a hemoglobin depletion period, four fruit juices comprised of (1) FS, (2) MDFP, (3) FS + AA, (4) MDFP + AA were produced and administered to a different group of rats (n = 18) over 21 days. During the repletion period, Fe balance, hemoglobin regeneration efficiency (HRE), relative bioavailability (RBV) and Fe tissue content were determined in the short, medium and long term. *Results* Fe absorption and bioavailability showed no significant differences between fortifying the fruit juice with FS or MDFP. The addition of AA to the juice enhanced Fe absorption during the long-term balance study within the same Fe source. HRE and Fe utilization increased after AA addition in both FS and MDFP groups in every period. *Conclusion* Fe absorption and bioavailability from MDFP were comparable to FS added to a fruit juice in rats. Further, the addition of AA enhanced Fe absorption in the long term, as well as Fe bioavailability throughout the repletion period regardless of the Fe source employed.

Key words iron bioavailability – ascorbic acid – ferrous sulfate – micronized dispersible ferric pyrophosphate – rats

Introduction

Food fortification with iron (Fe) has been recommended as one of the preferred approaches for preventing and eradicating Fe deficiency [34]. However, fortification with bioavailable Fe sources often presents multiple challenges in product acceptance, product shelf life, and effectiveness in terms of improving Fe status [11]. This is the case with water-soluble Fe compounds such as ferrous sulfate (FS), which is desirable used as food fortificant, but is not generalized because of sensory issues. Conversely, poorly water-soluble Fe compounds, such as ferric

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pyrophosphate and elemental Fe powders, although more stable in foods, tend to have low bioavailability [12]. A potential strategy for overcoming this problem is the reduction of the particle size of poorly water-soluble Fe compounds to increase their dissolution rate and thereby improve their bioavailability [20]. Studies carried out in humans [31] and animals [26] demonstrated that reducing the particle size of elemental Fe powders increased relative bioavailability (RBV) compared to FS. Recently, a micronized dispersible ferric pyrophosphate (MDFP) with a mean particle size of 0.5 μm, coated in monoglycerides and diglycerides to minimize aggregation, has been developed [21]. This compound has the additional advantage of being dispersible in aqueous solutions and can be used to fortify liquid foods or drinks. This Fe compound has also been reported to have a RBV in humans of 82 and 92% from a wheat-milk infant cereal and yogurt drink, respectively [5]. Although MDFP is a promising Fe fortificant, the potential influences of food matrix, food processing and absorption enhancers on its bioavailability are uncertain [20]. One strategy that is available for increasing the bioavailability of Fe fortificants and not causing adverse sensory changes in the chosen vehicles is the addition of ascorbic acid (AA) [15]. In the present work, fruit juice was selected as the food vehicle due to its low content in Fe absorption inhibitors and widespread consumption among the young and adult populations, making it an interesting choice for Fe fortification. Hence, the aim of our study was to compare Fe absorption and bioavailability from either MDFP or FS added to a fruit juice in the presence or absence of AA using the hemoglobin (Hb) repletion assay in rats.

Material and experimental methods

Chemicals

All chemicals were obtained from Sigma Chemicals unless stated otherwise. Water used in the preparation of solutions for rat tissue analysis, fecal and food samples was double deionized. Glassware and utensils were soaked in 10% HNO_3 overnight and rinsed three times with deionized water prior to use.

Experimental design

A full factorial 2^k experimental design was used to study the effect of Fe type (ferrous sulfate, FS or micronized dispersed ferric pyrophosphate, MDFP) and AA addition on Hb regeneration, Fe absorption and bioavailability in rats. The study was based on the animal diet (described below), depending on the chemical form of the supplemented Fe compound and the presence of AA.

Animal diet

Rats consumed an Fe-deficient diet (2.6 mg Fe/kg AIN-93G purified rodent diet) ad libitum for 28 days. The diet was prepared by Harland Interfauna Ibérica S.L. (Barcelona, Spain) and comprised of (g/kg): 315 cornstarch, 200 vitamin-free casein, 314.5 sucrose, 70 soybean oil, 50 cellulose, 35 Fe-free mineral mix (ferric citrate-free AIN-93G mineral mix), 10 vitamin mix (AIN-93VX), 3 L-cystine, and 2.5 choline bitartrate.

During the repletion period, all rats consumed the Fe-deficient AIN-93G diet. The Fe source was administered through fruit juice (50% fruit content), which consisted of pineapple (47%) and passion fruit (3%) (Hero Spain, S.A, Murcia, Spain) fortified with either ferrous sulfate (FS, Merck) or micronized dispersible ferric pyrophosphate, coated in monoglycerides and diglycerides (MDFP, SunActive Fe™), with a mean particle size of 0.5 μm, both with or without ascorbic acid (AA) supplementation. The AA was incorporated into the fruit juice to obtain a final concentration of 40 mg/100 ml as determined by HPLC analysis after extraction with meta-phosphoric acid according to Klopotek et al. [16]. Thus, four different juices referred as: (1) FS, (2) MDFP, (3) FS + AA, (4) MDFP + AA were administered to rats. Additionally, another group of rats (referred as control group in Table 4 footnote) were fed only the AIN-93G diet in order to compare Fe content in these rats' tissues with that found in the test groups. The rate of pineapple:passion fruit in the fruit juice remained constant over the 21 days of the study. New bottles belonging to the same batch were opened everyday to avoid degradation of the ingredients. The nutritional composition of the fruit juice was (g/100 ml): 0.2 proteins, 5.9 carbohydrates (5.0 sugars), 0.1 fat (0.0 saturated fatty acids) and 0.5 fiber. The Fe content in both the Fe-deficient diet (2.6 mg Fe/kg) and the fortified fruit juices (46 mg Fe/kg) was verified by atomic absorption spectroscopy.

Hemoglobin repletion test in rats

The study was approved by the bioethics committee of University of Murcia and the rats were housed and cared for in accordance with the Spanish law RD 1201/2005, 10 October, Protection of animals used for

experimental purposes. The hemoglobin (Hb) depletion-repletion method was used to determine Fe bioavailability and utilization [19, 35]. Fe balance and Fe percentage of absorption were evaluated during the repletion period.

Male weanling Sprague-Dawley rats (n = 72; Harlan Interfauna Ibérica, SL, Barcelona, Spain), at the age of 21–23 days, were housed in groups of 2–3 rats in plastic cages under the following conditions: 22 ± 2°C temperature, 50% relative humidity, and a 12 h light-dark cycle (light exposure from 8:00 a.m. to 8:00 p.m.). After a 28-day depletion period using an Fe-deficient diet and deionized water ad libitum, the rats were weighed and a blood sample was obtained from the tail vein to measure Hb initial concentration. Hb concentration was measured in a blood counter (Veterinary Animal Blood Counter, ABX Diagnostics, Montpellier, France). The Hb concentration after the depletion period was 7.0 ± 0.37 g/dl and the rats were assigned to four groups of 18 animals according to their weight at the beginning of the repletion period. Each group was fed with the same Fe-deficient diet (AIN–93G) and one of the four experimental fruit juices during the 21-day repletion period. The Fe balance study was performed on three successive days on three occasions during the experiment: on days 1–3 (short-term balance), days 10–12 (medium-term balance) and days 18–20 (long-term balance). In brief, six rats of each group were housed in stainless steel metabolic cages under the same conditions described for the depletion period; the remaining animals were housed in a ratio of two per cage (wire-bottomed to limit coprophagy). During the balance study, the rats' weight and food intake were recorded daily. Fecal samples were also collected daily, separated from spilt food with a nylon sieve and stored at −20°C until used for further analysis. The next day of each balance study (days 4, 13 and 21), rats were killed, then blood samples were collected for Hb response analysis; the heart, liver, spleen, kidney and proximal small intestine (~20 cm) were removed for further analysis.

Calculations

Hb gain was determined as the difference between the Hb value on days 4, 13 or 21 and the Hb value on day 1. Percentage of bioavailability was calculated as hemoglobin regeneration efficiency (HRE) (%): 100 × [mg Fe Hb (final) − mg Fe Hb (initial)]/mg Fe consumed, where initial corresponds to day 1, and final to day 4, 13 or 21. The Fe content of Hb (mg) was calculated assuming a total blood volume of 6.7% of the rat body weight, and an average Fe content of Hb of 0.335 [29]: [body weight (g) × Hb (g/l) × 6.7 × 0.335]/10,000. Fe utilization (mg) was calculated according to the following equation: [HRE (%) × Fe in fruit juice (%)]/100. Relative bioavailability (RBV) was determined as follows: 100 × (HRE from each group) / HRE from fruit juice fortified with FS. Fe balance (mg) was calculated according to the following equation: (mineral intake − fecal mineral excretion), whereas percentage of Fe absorption (%) was determined as follows: 100 × (Fe absorption/mineral intake).

Analysis

After overnight food deprivation at the end of each balance period, the rats were first anesthetized with isoflurane, and then blood was collected from the cava vein into heparinized syringes for immediate Hb analysis in a blood counter (Veterinary Animal Blood Counter, ABX Diagnostics, Montpellier, France).

Fe concentration in the depletion diet, fruit juices, fecal samples and tissues (liver, spleen, heart, kidney and proximal small intestine) were determined by atomic absorption spectrophotometry. Feces and fruit juices were previously dried, while tissues were freeze-dried, weighed and milled to a fine powder. Samples were dry-ashed in a furnace oven at 550°C over 12 h, then dissolved in 5 ml HCl fuming 37% and 2 ml HNO_3 65% and heated on an electric hot plate until dried. The resulting mineral solutions were brought up to 50 ml with deionized water. Fe was measured using a Perkin-Elmer atomic absorption spectrophotometer model 3100 (Norwalk, CT) with an air-acetylene flame at 248.3 nm. The acids (Suprapur® quality) and mineral standard solution (Titrisol®) used were obtained from Merck (Darmstadt, Germany). The Fe concentration in tissue samples was expressed as μg Fe/g of dry tissue sample and used as an index of rat Fe status.

Statistical analysis

Results obtained in the animal study were expressed as means ± SEM of six determinations. Data analyses included a 2-way ANOVA test at a significance level of $P < 0.05$ and a by-couples comparison multiple test using a Tukey analysis to calculate the effects of the Fe source and the addition of AA to fruit juice on the different responses considered. To measure the strength of the relationships among the different parameters measured to obtain Fe absorption and Fe bioavailability with the Fe total intake of rats during the repletion period, a correlation analysis using the Pearson's correlation coefficient was conducted. Statistical analyses were carried out using the SPSS 11.5 software package (Chicago, IL, USA).

Table 1 Weight gain, food and juice intake and iron (Fe) balance in rats fed with fruit juice with or without ascorbic acid (AA) supplemented either fortified with ferrous sulfate (FS) or with micronized dispersible ferric pyrophosphate (MDFP) during repletion period

Parameters	FS	MDFP	FS + AA	MDFP + AA	Fe effect	AA effect	Interaction
Short-term balance (days 1–3)							
Weight gain (g)	10.30 ± 4.33	12.47 ± 2.65	12.60 ± 3.93	12.82 ± 2.44^{b}	NS	NS	NS
Food intake (g)	23.25 ± 2.33	21.98 ± 6.07	38.52 ± 3.84$^{b/*}$	25.55 ± 1.46^{b}	NS	0.007	0.002
Juice intake (ml)	130.00 ± 24.55	199.60 ± 38.28	93.17 ± 22.71^{b}	77.17 ± 10.52$^{b/*}$	NS	0.0001	0.031
Fe intake (mg)	7.93 ± 0.95^{b}	11.16 ± 3.58	4.32 ± 0.69$^{b/*}$	4.28 ± 0.38$^{b/*}$	NS	0.0001	NS
Fe excreted (mg)	1.47 ± 0.15^{b}	1.54 ± 0.68^{c}	1.37 ± 0.06^{c}	1.25 ± 0.09^{c}	NS	NS	NS
Fe balance (mg)	6.45 ± 0.97^{b}	9.62 ± 1.77	2.96 ± 0.68*	3.02 ± 0.29*	NS	0.0001	NS
Fe absorption (%)	80.33 ± 3.85^{a}	85.65 ± 3.43^{a}	72.06 ± 5.17^{a}	70.50 ± 0.67$^{a/*}$	NS	0.002	NS
Medium-term balance (days 10–12)							
Weight gain (g)	13.70 ± 6.43	17.35 ± 2.40	17.23 ± 3.20	20.83 ± 3.84^{a}	NS	NS	NS
Food intake (g)	33.74 ± 3.36	36.32 ± 2.66	47.82 ± 2.92$^{a/*}$	44.13 ± 3.31^{a}	NS	0.010	NS
Juice intake (ml)	201.40 ± 43.59	263.33 ± 22.71	209.33 ± 24.10^{a}	95.50 ± 16.96$^{ab/*}$	NS	0.003	0.006
Fe intake (mg)	12.09 ± 0.60^{a}	13.67 ± 1.13	10.99 ± 1.27^{a}	5.35 ± 0.79 $^{ab/*}$	NS	0.003	0.006
Fe excreted (mg)	3.89 ± 0.39^{ab}	4.20 ± 0.61^{b}	5.88 ± 0.54$^{a/*}$	2.03 ± 0.19$^{b/*}$	0.005	0.05	0.005
Fe balance (mg)	8.20 ± 0.61^{a}	9.47 ± 0.95	5.12 ± 0.87*	3.31 ± 0.62*	NS	0.0001	0.05
Fe absorption (%)	67.74 ± 3.33^{b}	69.25 ± 3.24^{b}	49.21 ± 1.25$^{b/*}$	62.79 ± 1.81$^{a/*}$	NS	0.019	NS
Long-term balance (days 18–20)							
Weight gain (g)	22.78 ± 4.22	16.07 ± 3.78	10.09 ± 2.69*	4.89 ± 1.46$^{c/*}$	NS	0.003	NS
Food intake (g)	33.13 ± 3.64	21.20 ± 4.89	48.67 ± 1.58$^{a/*}$	41.34 ± 3.16$^{a/*}$	NS	0.0001	NS
Juice intake (ml)	218.00 ± 93.09	183.75 ± 60.26	123.75 ± 41.57$^{b/*}$	131.38 ± 12.17$^{a/*}$	NS	0.013	NS
Fe intake (mg)	10.81 ± 2.29^{b}	12.46 ± 1.99	5.64 ± 0.55$^{b/*}$	7.12 ± 0.64$^{a/*}$	NS	0.002	NS
Fe excreted (mg)	6.27 ± 1.31^{a}	6.66 ± 0.45^{a}	2.55 ± 0.12$^{b/*}$	3.26 ± 0.33$^{a/*}$	NS	0.0001	NS
Fe balance (mg)	4.54 ± 1.01^{b}	5.81 ± 1.62	3.09 ± 0.46	3.99 ± 0.44	NS	NS	NS
Fe absorption (%)	41.90 ± 1.34^{c}	44.88 ± 6.06^{c}	53.24 ± 3.54$^{b/*}$	56.48 ± 4.55$^{b/*}$	NS	0.019	NS

*Asterisks within the same row for every balance period show significant differences ($P < 0.05$) between FS and FS + AA groups, and MDFP and MDFP + AA groups
$^{a-c}$Different letters in superscript within the same column show significant differences ($P < 0.05$) among the three balance studies for each fruit juice studied
Fe effect statistical significance shown between supplementation with FS or MDFP by ANOVA test ($P < 0.05$), *AA effect* statistical significance shown between supplementation with FS or FS + AA, and MDFP or MDFP + AA by ANOVA test ($P < 0.05$), *Interaction* statistical significance shown between supplementation with FS + AA and MDFP + AA by ANOVA test ($P < 0.05$)

Results

Iron balance

Fe balance, as well as weight gain, food and juice intake in rats fed the four test fruit juices over the short, medium and long term are shown in Table 1. The different sources of Fe, FS as opposed to MDFP, had no significant effect on weight gain, food, juice or Fe intake, Fe balance and absorption during the short, medium and long-term balance studies. It was only observed that there was a significantly ($P < 0.005$) higher Fe excretion in the MDFP group during the medium-term balance study. No significant differences were found in the percentage of Fe absorption between Fe sources (FS and MDFP) for any balance period. On the other hand, the addition of AA to the fruit juices enhanced Fe absorption during the long-term balance study within the same Fe source (from 41.90 to 53.24% in the FS group and from 44.88 to 56.48% in the MDFP group), as shown in Table 1. However, no significant differences were found in Fe absorption in the three balance studies when comparing the FS + AA group to the MDFP + AA group.

Iron bioavailability

Table 2 shows Fe bioavailability in rats fed the four test fruit juices in the short, medium and long term at the end of each balance study. With regard to the Fe bioavailability calculations, it must be pointed out that each period began on the same day (day 1) but finished 3 days later for the short-term period, 12 days later for the medium-term period and 20 days later for the long-term period. Thus, the values obtained for weight gain and Fe intake were different from those observed in Table 1. In general, no significant differences were found due to the different source of Fe added to the juices for any of the parameters measured. The addition of AA to the fruit juice did not significantly change Fe Hb gain in either the FS or MDFP group, but significantly ($P < 0.006$) decreased Hb gain in both groups in the long-term study. In addition, HRE and Fe utilization significantly ($P < 0.001$) rose when AA was added to both the FS and MDFP groups in every balance study. However, no significant differences were found between the FS + AA and MDFP + AA groups for Hb gain, Fe Hb gain, Fe bioavailability (%HRE) or Fe utilization. In general, RBV was higher in

Table 2 Hemoglobin and iron (Fe) responses in rats fed with fruit juice with or without ascorbic acid (AA) supplemented either fortified with ferrous sulfate (FS) or with micronized dispersible ferric pyrophosphate (MDFP) during repletion period

Parameters	FS	MDFP	FS + AA	MDFP + AA	Fe effect	AA effect	Interaction
Short-term (day 4)							
Weight gain (g)	10.30 ± 4.33^{c}	12.47 ± 2.65^{c}	12.60 ± 3.93^{c}	12.82 ± 2.44^{c}	NS	NS	NS
Fe intake (mg)	7.93 ± 0.95^{c}	11.16 ± 3.58^{c}	4.32 ± 0.69$^{c/*}$	4.28 ± 0.38$^{c/*}$	NS	0.0001	NS
Hb gain (g/dl)	3.80 ± 0.17^{c}	3.60 ± 0.59^{b}	3.25 ± 0.29^{b}	3.93 ± 0.32^{b}	NS	NS	NS
Fe Hb gain (mg)	1.39 ± 0.18^{b}	1.43 ± 0.28^{b}	2.06 ± 0.22^{b}	1.58 ± 0.14^{b}	NS	NS	NS
HRE[1] (%)	20.55 ± 4.27^{a}	14.80 ± 3.17^{a}	40.67 ± 6.76$^{a/*}$	39.42 ± 5.53$^{a/*}$	NS	0.001	NS
Fe utilization (mg)	1.01 ± 0.21^{a}	0.73 ± 0.15^{a}	2.00 ± 0.66$^{a/*}$	1.94 ± 0.61$^{a/*}$	NS	0.001	NS
RBV[2] (%)	100	79	198	192			
Medium-term (day 13)							
Weight gain (g)	38.05 ± 3.96^{b}	42.12 ± 6.97^{b}	68.35 ± 1.91$^{b/*}$	69.38 ± 1.86$^{b/*}$	NS	0.0001	NS
Fe intake (mg)	27.47 ± 4.78^{b}	30.33 ± 2.72^{b}	28.04 ± 1.91^{b}	21.45 ± 0.86$^{b/*}$	NS	0.025	0.038
Hb gain (g/dl)	6.50 ± 0.18^{b}	6.97 ± 0.34^{a}	6.58 ± 0.32^{a}	6.64 ± 0.25^{a}	NS	NS	NS
Fe Hb gain (mg)	4.51 ± 0.53^{a}	4.13 ± 0.23^{a}	5.72 ± 0.42^{a}	4.98 ± 0.40^{a}	NS	NS	NS
HRE (%)	16.85 ± 5.06^{ab}	13.79 ± 2.79^{a}	20.51 ± 1.12^{b}	23.36 ± 2.14$^{b/*}$	NS	0.0001	NS
Fe utilization (mg)	0.83 ± 0.25^{ab}	0.68 ± 0.14^{a}	1.01 ± 0.14^{b}	1.15 ± 0.26$^{b/*}$	NS	0.0001	NS
RBV (%)	100	82	122	139			
Long-term (day 21)							
Weight gain (g)	80.50 ± 15.73^{a}	64.30 ± 7.21^{a}	99.36 ± 6.20^{a}	93.90 ± 3.70$^{a/*}$	NS	0.010	NS
Fe intake (mg)	56.78 ± 4.58^{a}	53.04 ± 5.95^{a}	52.22 ± 1.18$^{a/*}$	50.13 ± 1.50^{a}	NS	NS	NS
Hb gain (g/dl)	7.67 ± 0.53^{a}	8.25 ± 0.66^{a}	6.33 ± 0.15^{a}	6.65 ± 0.18$^{a/*}$	NS	0.006	NS
Fe Hb gain (mg)	5.64 ± 0.44^{a}	4.32 ± 0.45^{a}	6.17 ± 0.23^{a}	5.42 ± 0.14^{a}	NS	NS	NS
HRE (%)	10.02 ± 1.96^{b}	8.09 ± 0.88^{b}	11.85 ± 0.51^{c}	10.91 ± 0.56$^{c/*}$	NS	0.014	NS
Fe utilization (mg)	0.49 ± 0.09^{b}	0.40 ± 0.04^{b}	0.58 ± 0.07^{c}	0.54 ± 0.07$^{c/*}$	NS	0.014	NS
RBV (%)	100	81	118	109			

*Asterisks within the same row for every term show significant differences ($P < 0.05$) between FS and FS + AA groups, and MDFP and MDFP + AA groups
[a-c]Different letters in superscript within the same column show significant differences ($P < 0.05$) among the terms for each fruit juice studied
Fe effect statistical significance shown between supplementation with FS or MDFP by ANOVA test ($P < 0.05$), *AA effect* statistical significance shown between supplementation with FS or FS + AA, and MDFP or MDFP + AA by ANOVA test ($P < 0.05$), *Interaction* statistical significance shown between supplementation with FS + AA and MDFP + AA by ANOVA test ($P < 0.05$)
[1]*HRE* haemoglobin regeneration efficiency, HRE = 100 × [mg Fe Hb (final) − mg Fe Hb (initial)]/mg Fe consumed. Where, initial = day 1, and final = day 4, 13 or 21
[2]*RBV* relative bioavailability, RBV = 100 × (HRE from each group)/HRE from citric fruit juice fortified with FS

groups fed FS-containing juice than in those fed MDFP-containing juice. The addition of AA to the juices increased RBV regardless of the Fe source in every balance period.

Furthermore, the data from Table 2 indicates that while Fe intake significantly ($P < 0.05$) increased in all groups throughout the depletion period. Hb gain and Fe Hb gain also increased from the short-term to the medium term balance but not significant changes were determined with respect to the long-term balance in all four groups studied (except for Hb gain in group fed FS). Thus, a dose-response relationship can be established between Fe intake and Hb gain, and between Fe intake and Fe Hb gain. This response is summarized in the form of correlation coefficients among Fe intake, Hb gain and Fe Hb gain in Table 3. In all cases there was a high significant ($P < 0.001$) positive correlation (r = 0.678–0.872) between Fe intake and the other variables (Hb gain and Fe Hb gain) during repletion period regardless of the type of Fe source used and whether or not ascorbic acid was added into the fruit juice.

Tissue iron concentration

In Table 4 the Fe content (mg/g DW) in the liver, spleen, kidney, heart and small intestine of rats fed the four fruit juices evaluated in this work is shown. To better explain the results from the Fe concentration in tissues obtained in the four test groups, data from the medium-term period were not incorporated into Table 4, since no significant differences existed with respect to the long-term period. Furthermore, to compare the results obtained between short- and long-term periods, we analyzed Fe concentration from the same tissues in the control group (Table 4 footnote). We also confirmed that FS and MDFP groups reached comparable values with respect to the control group in these two periods. The Fe source showed that Fe concentration in the kidney and heart was significantly ($P < 0.015$ and $P < 0.017$) higher in the MDFP group (2.14 and 2.83 mg Fe/g, respectively) than in the FS group (1.40 and 1.51 mg Fe/g, respectively). The addition of AA did not increase Fe concentration in any of the tissues analyzed in either group (FS or MDFP). In fact, Fe concentration in the spleen de-

Table 3 Correlation between Fe Hb gain and Hb gain with Fe total intake during the repletion period

	FS		MDFP		FS + AA		MDFP + AA	
	Hb gain	FeHb gain	Hb gain	FeHb gain	Hb gain	FeHb gain	Hb gain	FeHb gain
Fe intake	$r = 0.872$ $N = 14$ $P = 0.0001$	$r = 0.833$ $N = 14$ $P = 0.0001$	$r = 0.782$ $N = 15$ $P = 0.001$	$r = 0.810$ $N = 15$ $P = 0.0001$	$r = 0.678$ $N = 16$ $P = 0.004$	$r = 0.833$ $N = 16$ $P = 0.0001$	$r = 0.731$ $N = 18$ $P = 0.001$	$r = 0.771$ $N = 18$ $P = 0.0001$

FS group fed fruit juice containing ferrous sulfate, *MDFP* group fed fruit juice containing micronized dispersible ferric pyrophosphate, *FS + AA* group fed fruit juice containing ferrous sulfate and supplemented with ascorbic acid, *MDFP + AA* group fed fruit juice containing micronized dispersible ferric pyrophosphate and supplemented with ascorbic acid

creased significantly ($P < 0.036$) during the short-term period, and in the small intestine it decreased significantly ($P < 0.0014$) in both the short- and long-term periods. Again, the comparison of the FS + AA and MDFP + AA groups did not show significant differences regarding Fe concentration in tissues in either the short- or long-term period. However, Fe concentration in the liver, spleen and small intestine was significantly higher ($P < 0.05$) in the MDFP + AA group in the long-term period with respect to the short-term period. Fe content in the liver and spleen was significantly ($P < 0.05$) higher in the MDFP + AA group in the long-term period with respect to the control group. However, only in the liver did the Fe content increase significantly ($P < 0.05$) in the FS + AA group as compared to the control group.

Discussion

In this study, we investigated the effect of AA on the Fe absorption and bioavailability of MDFP in a liquid matrix (fruit juice), using FS as reference fortificant. MDFP is a promising Fe fortificant, but the influence of the food matrix, food processing, and the absorption of enhancers on MDFP bioavailability is uncertain [20]. MDFP was added to a fruit juice due to the liquid nature of the matrix and the low content of Fe inhibitors. In addition, in a previous work we proved that the bioaccesibility of MDFP added to the same fruit juice was very similar to FS (27.9 and 28.2%, respectively) [10].

Iron balance

The Fe absorption was similar in the FS and MDFP groups after short-, medium- and long-term Fe intake, but it diminished from the short- to the long-term study. It is known that an increase of Fe status leads to a decrease in Fe absorption, which is clearly reflected in our study by the lower Fe absorption in days 15–18, when compared to days 1–4 and 8–11. The relationship between food intake and Fe status is

Table 4 Iron (Fe) concentration (mg/g DW) in different tissue samples in rats fed with fruit juice with or without ascorbic acid (AA) supplemented either fortified with ferrous sulfate (FS) or with micronized dispersible ferric pyrophosphate (MDFP) during repletion period

Tissue	FS	MDFP	FS + AA	MDFP + AA	Fe effect	AA effect	Interaction
Short-term (day 4)							
Liver	$0.93 \pm 0.32^{(1)}$	0.52 ± 0.27	0.74 ± 0.25	0.42 ± 0.12^{b}	NS	NS	NS
Spleen	6.54 ± 2.68	$9.04 \pm 1.65^{(1)}$	3.44 ± 0.45	3.43 ± 1.38^{b}	NS	0.036	NS
Kidney	1.40 ± 0.35	2.14 ± 0.24	1.04 ± 0.08^{a}	1.67 ± 0.13	0.015	NS	NS
Heart	1.51 ± 0.30	2.83 ± 0.52	1.39 ± 0.21	2.18 ± 0.20	0.017	NS	NS
Small intestine	$0.85 \pm 0.31^{(1)}$	$0.96 \pm 0.35^{(1)}$	0.18 ± 0.04	0.17 ± 0.02^{b}	NS	0.014	NS
Long-term (day 21)							
Liver	$1.03 \pm 0.15^{(1)}$	0.69 ± 0.17	$0.87 \pm 0.14^{(1)}$	$0.91 \pm 0.13^{a\ (1)}$	NS	NS	NS
Spleen	5.40 ± 1.68	$7.10 \pm 1.57^{(1)}$	5.35 ± 2.67	$8.13 \pm 0.80^{a\ (1)}$	NS	NS	NS
Kidney	1.36 ± 0.03	1.70 ± 0.45	$0.75 \pm 0.06^{b/*\ (1)}$	1.30 ± 0.20	NS	NS	NS
Heart	1.63 ± 0.17	2.15 ± 0.46	1.43 ± 0.04	1.46 ± 0.20	NS	NS	NS
Small intestine	$0.56 \pm 0.22^{(1)}$	$0.91 \pm 0.19^{(1)}$	0.15 ± 0.04	$0.37 \pm 0.03^{a\ (1)}$	NS	0.013	NS

Iron concentration (mg/g DW) of different tissues in control group: Liver (0.45 ± 0.03), Spleen (3.66 ± 0.39), Kidney (1.56 ± 0.18), Heart (2.00 ± 0.25), Small intestine (0.11 ± 0.01)

*Asterisks within the same row for every term show significant differences ($P < 0.05$) between FS and FS + AA groups, and MDFP and MDFP + AA groups

[a-c]Different letters in superscript within the same column show significant differences ($P < 0.05$) among the terms for each fruit juice studied

[(1)]Significant ($P < 0.05$) differences with regard to control group

Fe effect statistical significance shown between supplementation with FS or MDFP by ANOVA test ($P < 0.05$), *AA effect* statistical significance shown between supplementation with FS or FS + AA, and MDFP or MDFP + AA by ANOVA test ($P < 0.05$), *Interaction* statistical significance shown between supplementation with FS + AA and MDFP + AA by ANOVA test ($P < 0.05$)

also supported by the high linear correlation coefficients observed between Fe intake and Fe balance in the FS and MDFP groups ($r = 0.729$; $P < 0.01$, in the MDFP group and $r = 0.634$, $P < 0.05$ in the FS group), which is in agreement with results from other authors [1, 23].

In the present study, the addition of AA affected Fe absorption in both the FS and MDFP groups. Derman et al. [3] reported similar results when AA was added to infant cereals fortified with FS, ferric pyrophosphate or ferric ammonium citrate. This could be related to the solubility rate of both Fe compounds. FS is a highly water soluble Fe compound, whereas in poorly water soluble Fe compounds such as MDFP, Fe is dissolved in the common pool at a slower rate. Therefore, depending on the Fe source, the digestion and release of nonheme Fe into the common pool or its subsequent absorption can be strongly influenced by the effect of the matrix [9]. The fact that AA was added to the fruit juice at a 2.8:1 molar ratio in relation to Fe could also explain the results obtained. Indeed, several authors reported that for AA to improve Fe absorption in low phytate-content products, the vitamin C:Fe molar ratio must be 2:1 [15, 17].

Iron bioavailability

Our results showed that the percentage of Fe bioavailability expressed as HRE for the MDFP group was similar to that of the FS group in all periods of the balance study. This is in accordance with a previous study in which, after 2 weeks of Fe fortification, the HRE value of MDFP was similar to FS [22].

We observed that Fe intake was positively correlated with both Hb gain ($r = 0.872$, $P < 0.0001$ for the FS group, and $r = 0.782$, $P < 0.001$ for the MDFP group) and FeHb gain ($r = 0.833$, $P < 0.0001$ for the FS group and $r = 0.810$ $P < 0.0001$ for the MDFP group). Therefore, the Fe provided by FS and MDFP was positively and equally incorporated into the Hb, and was also inversely correlated with HRE ($r = -0.803$, $P < 0.001$ for the FS group and $r = -0.774$, $P < 0.001$ for the MDFP group). This last correlation was also observed by Buchowski [1], who concluded that HRE was high in anemic rats and inversely related to Fe intake.

When comparing Fe intake with the Hb regeneration parameters, namely Hb gain and Fe Hb gain, the latter in general gave slightly better correlation coefficients than the former for both dietary groups (Table 3). This could be due to the fact that Fe Hb gain takes into consideration the differences in weight gain, and thus in the expansion of blood volumes during the regeneration period [25]. As shown in Table 2, the Hb gain and Fe Hb gain was similar between the medium and long-term balance studies (except Hb gain for FS group). This effect occured regardless of Fe intake and compound because the daily Fe intake during these periods was about 3 mg. This indicates a recovery from Fe deficiency anemia through the intake of fruit juice fortified with either FS or MDFP, with or without AA. However, the best parameter that described the recovery from Fe deficiency anemia was HRE. Aditionally, the suplementation with AA enhanced HRE in each balance period (Table 2).

Moreover, RBV in the MDFP group could be considered as high (79, 82 and 81% the in short-, medium- and long-term repletion periods, respectively), if compared to FS, regarded as a reference (RBV 100%). This observation might be related to the extremely small particle size of the Fe compound, which is approximately twenty times smaller than that of regular ferric pyrophosphate [5]. A previous rat Hb repletion study showed an RBV of 78 and 104% in commercial ferric pyrophosphate and MDFP, respectively, compared to FS [22]. However, it is unclear whether the high RBV of the ferric pyrophosphate compound is due only to its small particle size, or whether the surrounding emulsifiers may also play an important role [32]. On the other hand, the effect of food industry processing on RBV is variable and difficult to predict. It is possible that the pasteurization process applied to the fruit juice increased RBV, most likely because of the solubilization of the ferric pyrophosphate. This has been reported previously in relation to a sterilized liquid formula fortified with MDFP, increasing RBV from 75 to 125% due to the heating process [20, 30].

Although AA and Na_2EDTA have repeatedly been shown to enhance Fe absorption from FS [2, 14, 18, 24], there have been few studies on the effect of AA on Fe bioavailability from MDFP. Previous studies have shown that the addition of AA enhanced the Fe bioavailability of other poorly soluble Fe compounds [6, 8, 9]. However, contradictory results have been also reported for MDFP and ferric pyrophosphate [6, 20]. Based on the results from the present study, and the method used to measure of relative Fe bioavailability, MDFP + AA reached an RBV similar to FS + AA in every period. If we consider FS as a reference (RBV 100%), our results confirm that the addition of AA to the fruit juice in a molar ratio of 2.8:1 with respect to Fe was enough to significantly increase RBV in both Fe sources even during the long-term period. AA has reducing and chelating properties that makes it the most efficient enhancer of nonheme Fe absorption when its stability in the vehicle is ensured. However, the magnitude of the Fe absorption improvement depends, not only on the molar ratio of AA to Fe, but also on the presence of other enhancers and inhibitors

(phytate and polyphenols) in the fortified food or meal [28]. Hurrell [13] recommended an AA:Fe molar ratio of 2:1 to enhance the absorption of soluble Fe compounds in milk products and low-phytate content foods, but a ratio of at least 4:1 should be used in foods with high phytate or phenolics content.

Tissue heme iron concentration

Anemia is a useful index of the severity of Fe deficiency, but many cases of Fe deficiency are more related to a deficiency of the Fe tissue stores. The liver and spleen are the main Fe storage tissues in the organism and, therefore, the liver and (to a lesser extent) the spleen have been routinely used as indicators of body Fe status in rats [4, 7, 33]. In this respect, we observed that the liver Fe concentration in the FS and MDFP groups was similar or higher than that found in the control group (0.45 mg/g). Although the administration of AA did not increase liver Fe concentration as compared to the AA-free groups, it was larger than the liver Fe concentration in the control group. This could be related to the reducing power of AA, which makes it capable of releasing Fe from ferritin and the reticulo endothelial cells to transferrin, increasing the Fe bioavailability and preventing Fe overload in tissues [27].

In conclusion, our results suggest that MDFP is an ideal compound with high bioavailability for Fe fortification in fruit juices. Furthermore, AA added at a 2.8:1 molar ratio (relative to Fe) enhanced Fe absorption from MDFP in the long term, whereas Fe bioavailability increased from the beginning of the daily consumption of MDFP-fortified juice.

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