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Iron Bioavailability from Pate Enriched with Encapsulated Ferric Pyrophosphate or Ferrous Gluconate in Rats

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Fortifying food with iron has been widely studied as a strategy to prevent iron deficiency anaemia. This work comparatively assessed the bioavailability of two forms of iron, ferrous gluconate or ferric pyrophosphate encapsulated in liposomes (lipofer®), when used as fortificants in meat pate. Three groups of growing rats consumed during 28 days either a control diet (AIN-93G), or two diets prepared with enriched pate as the unique source of iron and fat. Body weight and diet intake were measured weekly, and during the last week faeces were collected. On day 28 animals were sacrificed, livers and spleens were removed and stored. Haemoglobin and total iron binding capacity (TIBC) were determined. There were not significant differences among the three groups in body weight and apparent iron absorption, although food intake in the two pate groups was significantly higher compared to the control group. There were not differences in liver and spleen iron content and concentration, neither in haemoglobin and TIBC values. These results indicated that iron bioavailability of pate enriched with ferrous gluconate or ferric pyrophosphate encapsulated in liposomes was similar, and thus both sources of iron are good candidates to be used as fortifiers in meat based products. The present outcome needs to be validated in humans.

Key Words: encapsulated ferric pyrophosphate, ferrous gluconate, iron bioavailability, enriched pate

INTRODUCTION

Iron deficiency anaemia is a major health problem, affecting primarily menstruating women, infants and children, with high prevalence rates worldwide (WHO/UNICEF/UNU, 1998; WHO, 2001). One of the causes of nutritional anaemia is that the amount of iron absorbed is insufficient to meet body's requirements. This insufficiency may be due to inadequate iron intake from food and to low bioavailability (WHO, 2001). Non-heme iron comprises 85–95% of the iron in a typical Western diet, however its absorption is low and dependent on other dietary constituents (Cook, 1990). Non-heme dietary enhancers and inhibitors of iron absorption have been widely studied (Cook and Monsen, 1976; Hallberg *et al*., 1978; Engelmann *et al*., 1998; Hurrell *et al*., 1999; Baech *et al*., 2003).

Food fortification with iron may be an effective strategy to prevent iron deficiency anaemia, although successful iron fortification of foods remains a challenge. Iron compounds to be used as food fortificants should be adequate to introduce bigger doses of iron in food without changing physical and chemical properties of foods, as well as their sensory properties (Hurrell, 2002), to allow appropriate food processing, and from consumer's point of view to be easily absorbed without causing digestive problems. Water-soluble, highly bioavailable iron compounds react with water in foods and often cause adverse sensory modifications, such as changes in colour. In contrast, poorly soluble iron compounds are more stable in foods but show low bioavailability.

Among the many iron-salts used for iron fortification, the latest which have been studied are: ferrous bisglycinate (Layrisse *et al*., 2000; Fox *et al*., 1998), ferrous fumarate (Davidsson *et al*., 2000), elemental iron (Roe and Fairweather-Tait, 1999; Hoppe *et al*., 2006), ferrous lactate (Kapsokefalou, 2005), being ferric pyrophosphate the most extensively investigated in the past 5 years (Hurrell, 2002; Fidler *et al*., 2004; Wegmüller *et al*., 2004). Ferric pyrophosphate is white and produces negligible color change nor causes sensory changes. Most commercial forms of ferric pyrophosphate have a relative bioavailability ≤50% of ferrous sulphate (Hurrell,

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2002). Ferrous gluconate relative availability is higher than that of ferric pyrophosphate, but may show potential adverse sensory changes (Hurrell, 2002).

Iron encapsulation may decrease unwanted sensory effects and reduce interactions of iron with other dietary components although the final outcome depends on the food matrix (Hurrell, 2002). Unacceptable colour changes have been observed in encapsulated ferrous sulphate and ferrous fumarate when added to low-grade salt (Wegmüller *et al*., 2003) and decreased iron bioavailability was observed by encapsulating iron pyrophosphate with palm oil (Wegmüller *et al*., 2004).

Meat is a good source of bioavailable iron. Pate is a ready-to-eat spreadable meat product and is consumed by different population groups, therefore it may be an adequate matrix for iron fortification.

This work was aimed at comparing iron bioavailability in growing rats fed enriched pate with two different iron compounds: ferric pyrophosphate encapsulated in liposomes and ferrous gluconate and testing if pate is potentially a good vehicle for iron.

MATERIAL AND METHODS

Diets

Total iron content in the enriched pates used was 15 mg/100 g. Non-enriched pate contains 5 mg Fe/100 g, of which 16% is heme-iron (Hallberg and Hulthen, 2000). The final heme-iron in the experimental pate diets represented 6% of total iron content.

Enriched pate with ferric pyrophosphate encapsulated in liposomes (Lipofer®) or ferrous gluconate were provided by the same manufacturer in cans (La Piara SA, Barcelona, Spain). Two diets were prepared with pate (231.8 g/kg of diet) as the unique source of iron and fat (32.27 mg/kg and 70 g/kg, respectively). Pate also provided 31.3 g of protein and 2.33 g of carbohydrates per kg of diet, as well as vitamin A (9 g/kg diet), phosphorous (463.6 mg/kg diet) and zinc (6.72 mg/kg diet). The prepared diets contained casein (168.7 g/kg) as source of proteins, corn starch (527.17 g/kg) and sacarose (100 g/kg) as the source of carbohydrates, and for vitamins, a mix provided by Dyets Inc. (Bethlehem, PA, USA) was added (AIN-93VX without vitamin A since pate provides it in excess). Control diet was AIN-93G (Reeves *et al*., 1993) provided by Dyets Inc (Bethlehem, PA, USA). The mineral mix was prepared and added to ferric pyrophosphate and ferrous gluconate diets, according to the mineral composition recommended in the AIN-93G diet (Reeves *et al*., 1993) using the same salts, except for iron, and taking into account that pate also contains phosphorous and zinc.

The theoretical content of protein, fat and carbohydrates in the prepared diets was the same as in the AIN-93G diet (200, 70 and 529.50 g/kg respectively).

Analytical iron contents of the three diets (mg/kg diet) were 58.8 ± 3 for control diet, 61.0 ± 3.1 for lipofer® diet and 60.0 ± 2.7 for gluconate diet (mean ± standard error of seven determinations).

Methods

Biological Assay

Twenty-four three-week-old weanling Wistar rats (half males and half females; initial body weight 40.5 ± 0.2 g) were obtained from Centro de Investigaciones Biomédicas (CSIC, Madrid) and housed in metabolic cages in an environmentally controlled room, maintained at 20–22°C, with a 12 h light-dark cycle and 55–70% humidity, during 28 days.

The animals were randomly assigned to the dietary treatments: control diet, Lipofer® diet or gluconate diet, and had free access to food and demineralised water (Milli-Q plus, Millipore Iberica, S.A. Madrid, Spain). During the experiment, body weight and food intake were monitored weekly. From day 21 through day 28 faeces were collected, dried, weighed and homogenized.

On day 28, animals were anaesthetized using ketamine (Ketolar®, Pfizer Inc., New York, USA) and xilacine (Rompun®, Bayer AG Laboratories, Leverkusen, Germany) in a ratio of 4:1, and blood was drawn by cannulation of the carotid artery into acid-washed (HNO_3 10%) plastic vials and allowed to clot. Liver and spleen were removed and weighed. All tissues were stored frozen at −20°C until iron analysis. Rats were handled in accordance with the current European regulations regarding laboratory animals throughout the study.

Analytical Techniques

Haemoglobin was determined in fresh blood by the cyanmethaemoglobin method (Biomerieux, Lyon, France), and total iron binding capacity (TIBC) in serum by the transferrin saturation method (Ferrimat Kit, Biomerieux, Lyon, France).

Pate, diets, faeces, livers and spleens were dry-ashed in a muffle furnace at 500°C. Ashes were dissolved in an acid solution ($HCl/HNO_3/H_2O$: 1/2/1; Suprapur, Merck, Darmstadt, Germany). Iron was determined in all samples by atomic absorption spectrophotometry (Perkin-Elmer 1100B Norwalk, CT, USA). A stock standard solution of iron (1 g/L) was prepared from Tritrisol (Merck, Darmstad, Germany) ($FeCl_3$ in 15% HCl, 1.000 g ± 0.002 g). Calibration solutions were prepared from the stock standard solutions by serial dilution with demineralised water (MilliQ plus). A blank solution was also used.

Bovine lyophilised Liver (certified reference material CRM 185; Community Bureau of Reference, Brussels, Belgium) yielded an iron value of 213 ± 2 µg/g

(mean ± SD of five determinations) (certified value 214 ± 5 µg/g).

Iron Absorption Related Indices

The following indices were calculated from the data obtained for the intake, faecal and urinary excretion of iron:

Apparent absorption = intake − fecal excretion
% A/I = (apparent absorption/intake) × 100
Food eficiency = weight gain/intake
Hepatosomatic index (%) = (liver weight/body weight) × 100
Spleen/body weight ratio (%) = (spleen weight/body weight) × 100

Statistical Analysis

The data were processed by a one way analysis of variance (ANOVA), and *post-hoc* Bonferroni tests were performed. For comparing body weight changes and food efficiency a repeated measures analysis was performed. The level of significance was established at $P \leq 0.05$. Data were processed with the Statistical Package for Social Sciences (SPSS 13.0 for windows, 2004).

RESULTS AND DISCUSSION

Rats body weight evolution was similar during the four week-long experiment in the three groups and all the animals reached comparable final body weights. However, food intake was significantly higher in the groups of rats consuming pate diets compared to control group ($P < 0.01$; $P < 0.05$; $P < 0.05$; for days 7–14, 14–21 and 21–28 respectively) (Table 1). Therefore, food efficiency was lower in the pate groups, reaching statistical significance from day 7 to day 14 ($P < 0.001$) (Table 1).

Pates enriched with the two sources of iron, ferric pyrophosphate encapsulated in liposomes and ferrous gluconate, were similar in colour and smell, and were well accepted by the rats.

In the AIN-93G diet, fat is provided by soybean oil, with a high content in polyunsaturated fatty acids (PUFA), mainly linoleic acid (54%), while pate content in PUFA was much lower, and contained (data provided by the manufacturer) 49% of monounsaturated fatty acids (MUFA, mainly oleic acid) and 35% of saturated fatty acids (SFA, mainly palmitic acid). Therefore, the pate used in the present study presented a more saturated lipid profile than the control diet which exclusively contained soybean oil.

Results of food intake are in agreement with previous reports indicating that saturated fat compared to polyunsaturated presents better sensory acceptability (Edionwe and Kies, 1993; Wistuba *et al.*, 2006). However, the higher food consumption observed in the two pate groups did not lead to higher body weight because the control diet, rich in linoleic and linolenic acids, is the most adequate diet for rats at a growing stage (Reeves *et al.*, 1993). Liver and spleen weights were also similar in the three groups (Table 2), as well as the hepatosomatic index (3.54 ± 0.09% for control group, 3.35 ± 0.11% for Lipofer® group and 3.41 ± 0.09% for gluconate group) and spleen/body weight ratio (0.32 ± 0.03% for control group, 0.31 ± 0.02% Lipofer® group and 0.27 ± 0.02% for gluconate group). Reports indicate that excessive

Table 1. Food intake, body weight and food efficiency in rats.

	Rats Diet[1]			
Sampling and Variables	Control	Ferric Pyrophosphate	Ferrous Gluconate	ANOVA
Days 1–7				
Weight (g) day 1	40.40 ± 0.68	40.58 ± 0.64	40.55 ± 0.49	NS
Food intake (g/day)	9.19 ± 0.32	9.79 ± 0.46	10.27 ± 0.48	NS
Food efficiency	0.47 ± 0.01	0.45 ± 0.02	0.42 ± 0.01	NS
Days 7–14				
Weight (g) day 7	70.63 ± 1.97	71.01 ± 1.43	70.23 ± 1.47	NS
Food intake (g/day)	13.38 ± 0.46[a]	15.87 ± 0.47[b]	15.84 ± 0.72[b]	<0.01
Food efficiency	0.41 ± 0.01[a]	0.35 ± 0.01[b]	0.34 ± 0.01[b]	<0.001
Days 14–21				
Weight (g) day 14	109.64 ± 4.07	110.08 ± 3.36	107.99 ± 3.28	NS
Food intake (g/day)	15.56 ± 0.82[a]	20.31 ± 1.46[b]	18.71 ± 1.29[b]	<0.05
Food efficiency	0.29 ± 0.03	0.22 ± 0.02	0.26 ± 0.03	NS
Days 21–28				
Weight (g) day 21	141.75 ± 7.79	141.00 ± 5.71	145.2 ± 4.72	NS
Food intake (g/day)	16.17 ± 1.13[a]	20.13 ± 1.08[b]	19.62 ± 1.18[b]	<0.05
Food efficiency	0.31 ± 0.04	0.29 ± 0.03	0.27 ± 0.03	NS
Weight (g) day 28	178.35 ± 13.92	182.70 ± 9.60	183.13 ± 9.54	NS

Values are mean ± SEM of eight animals per group. Values in the same arrow followed by different superscripts are statistically significant. NS = non-significant.

Table 2. Iron content in liver and spleen of rats.

	Liver[1]			Spleen[1]		
Groups	Weight (g)	Iron Content (mg)	Iron Conc. (μg/g)	Weight (g)	Iron Content (μg)	Iron Conc. (μg/g)
Control	6.32 ± 0.49	1.16 ± 0.22	199.9 ± 46.4	0.57 ± 0.05	187.1 ± 43.9	304.1 ± 52.1
Ferric pyrophosphate	6.18 ± 0.48	1.03 ± 0.09	175.6 ± 23.0	0.58 ± 0.07	142.8 ± 32.4	233.8 ± 19.4
Ferrous gluconate	6.28 ± 0.44	1.13 ± 0.07	184.8 ± 15.6	0.50 ± 0.05	127.6 ± 30.8	248.0 ± 35.5
ANOVA	NS	NS	NS	NS	NS	NS

[1]Values are mean ± SEM of eight animals per group. NS = non-significant.

intake of PUFA may induce hepatic fatty acid oxidation which causes hepatomegalia (Pérez-Granados *et al*., 1995; Hashimoto *et al*., 2006) which was not the case of the present investigation.

Iron intake during the last week of the assay was higher in the pate groups than in the control group ($P < 0.01$) due to the higher food intake, being significantly more elevated in the case of the ferric pyrophosphate group. However, there were no significant differences due to the iron salt. Differences in faecal, apparent iron absorption and %A/I between the three groups were not significant (Table 3). Accordingly, the iron content and concentration of the spleen and liver did not show differences depending on whether the rats consumed diets with or without pate, or the iron salt (Table 2). Haemoglobin values were 14.1 ± 0.3 g/100 mL for control group; 13.6 ± 0.1 g/100 mL for ferric pyrophosphate group and 14.0 ± 0.6 g/100 mL for ferrous gluconate group. TIBC levels were also similar in the three groups (4.2 ± 0.2 for control; 4.8 ± 0.2 for ferric pyrophosphate; 4.6 ± 0.3 for ferrous gluconate) and within the normal range.

These results indicated that iron bioavailability of pate enriched with ferrous gluconate or ferric pyrophosphate encapsulated in liposomes was similar. These results were in agreement with in vitro iron availability results using Caco-2 cells, that showed similar iron availabilities with Lipofer®, ferrous lactate and ferrous sulphate, which are chemically similar to ferrous gluconate (Kloots *et al*., 2004). Ferrous gluconate, sulphate and lactate belong to the group of freely water soluble iron compounds. Thanks to being highly soluble in water, they enter the common pool on non-heme iron completely upon digestion and present high bioavailability. Hurrell *et al*. (2002) reported that the average bioavailability of ferrous gluconate was twice as high as that from ferric pyrophosphate. This can be explained because ferric pyrophosphate is poorly soluble in dilute acid, never dissolves completely in gastric juice and shows low relative bioavailability.

The present results support that encapsulation of ferric pyrophosphate, as in Lipofer®, protects iron from forming non-absorbable compounds and improves iron bioavailability. When encapsulating, the effects on iron bioavailability depended on the thickness of the capsule and the coating material (Hurrell, 2002) as well as on the ratio capsule/substract (Wegmüller *et al*., 2004). Wegmüller *et al*. (2004) described that encapsulation with a ratio capsule/substract of 60 : 40 decreased relative bioavailability values (RBV), either when ferrous sulphate or micronized ferric pyrophosphate were used. In contrast, when ferrous sulphate was encapsulated using a 40 : 60 capsule/substrate ratio, its RBV was not affected. Consistently, Lipofer® used in this study presented a ratio of 30 : 70, which improved iron bioavailability resulting similar to that of ferrous gluconate.

Lipofer® is a complex of ferric pyrophosphate, with an average particle size of 7 μm, encapsulated with starch and lecithin. Wegmüller *et al*. (2004) also comparatively investigated in rats the influence of encapsulation and particle size on RBV of ferric pyrophosphate. They obtained that encapsulation with hydrogenated palm oil, maintaining the same particle size (2.5 μm), decreased RBV, and that reducing particle size to 0.5 μm almost reached maximum RBV (95%). In our case the particle size of 7 μm also yielded good bioavailability results.

To our knowledge this is the first study that shows that lipofer® included in a meat product constitutes an iron supplement of high bioavailability. In addition, it is generally well accepted by a wide range of population and depending on the food process applied can be

Table 3. Iron absorption in the three groups of rats.

	Iron[1] (mg/day)			
Groups	Ingested	Fecal	Absorbed	% A/I
Control	0.95 ± 0.06[a]	0.46 ± 0.03	0.49 ± 0.03	51.89 ± 1.54
Ferric pyrophosphate	1.23 ± 0.06[b]	0.63 ± 0.08	0.60 ± 0.10	48.20 ± 7.46
Ferrous gluconate	1.18 ± 0.06[ab]	0.59 ± 0.06	0.58 ± 0.05	49.79 ± 3.97
ANOVA	<0.01	NS	NS	NS

Values are mean ± SEM of eight animals per group. Values in the same column followed by different superscripts are statistically significant. NS = non-significant.

stored, either refrigerated or at room temperature, for long periods of time. Further studies are needed to validate these results in humans considering technological, cost-effectiveness and health aspects.

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