

Development of nutritional iron deficiency in growing male rats: haematological parameters, iron bioavailability and oxidative defence

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Abstract

Despite Fe deficiency having been widely studied, the sequence of events in its development still remains unclear. The aim of the present study was to elucidate the effects of nutritional Fe-deficiency development on haematological parameters, Fe bioavailability and the enzymes involved in oxidative defence in recently weaned male Wistar albino rats. Control (C) and Fe-deficient (ID) groups were fed the AIN-93 G diet with a normal Fe level (45 mg/kg diet) or with a low Fe level (5 mg/kg diet), respectively, for 20, 30 or 40 d. At day 20 serum Fe, serum ferritin and the saturation of transferrin decreased drastically, decreasing further in the course of Fe-deficiency development for the saturation of transferrin. The development of Fe deficiency did not affect plasma thiobarbituric acid-reactive substance production, or catalase (CAT) and glutathione peroxidase (GPx) activities in erythrocyte cytosol. Fe deficiency diminished hepatic Fe content and CAT and GPx activities in hepatic cytosol only at day the 20. However, in spite of the minor Fe deposits in the brain of ID rats, the CAT and GPx activities in the brain cytosolic fraction did not differ in any of the studied periods *v.* control rats. These results show that brain is a tissue that does not seem to depend on Fe levels for the maintenance of antioxidant defence mechanisms in the course of nutritional Fe deficiency.

Key words: Iron-deficiency development: Oxidative defence: Liver and brain: Growing rats

Infants aged 6–24 months constitute one of the groups at highest risk of Fe deficiency⁽¹⁾. Fe requirements are most likely to exceed Fe intake at two time periods in the lifecycle: the first 6–18 months of postnatal life and then, for girls, during adolescence. Fe deficiency during the first year of life occurs at a time point of rapid neural development, and when morphological, biochemical and bioenergetic alterations may all influence future functioning^(2,3). Fe deficiency has been considered an important risk factor for ill health⁽⁴⁾. Fe status in infancy is determined by four main factors^(4,5): the Fe the infant is born with (which is related to maternal Fe status), the infant's postnatal needs for Fe, the external sources of bioavailable Fe and Fe losses. Fe is essential for all tissues in a young child's developing body⁽⁶⁾. The brain is the most vulnerable during critical periods of development⁽⁷⁾. Fe is present in the brain from very early in life, when it participates in the neural myelination processes^(8,9), learning and interacting behaviours⁽¹⁰⁾, and it is necessary by enzymes involved in the synthesis of the neurotransmitters serotonin (tryptophan

hydroxylase) and dopamine (tyrosine hydroxylase)⁽¹¹⁾. There is scant information available on the influence of Fe-deficiency development over enzymes related to oxidative defence. The objective of present study was to investigate the development of nutritional Fe deficiency (20, 30 or 40 d) in the early stages of life on oxidative defence in tissues such as the brain and liver in growing male rats. Moreover, in the present study, haematological parameters and Fe bioavailability were also investigated in order to know how Fe status changes during the induction of Fe deficiency.

Materials and methods

Animals

In the present study, forty-eight male Wistar albino rats, aged 3 weeks weighing about 40–46 g, purchased from the University of Granada Laboratory Animal Service were used. Animal care procedures and experimental protocols were approved by the Ethics Committee of the

Abbreviations: ADC, apparent digestibility coefficient; CAT, catalase; ID, Fe deficient; GPx, glutathione peroxidase; SOD, superoxide dismutase; TRARS, thiobarbituric acid-reactive substances.

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University of Granada in accordance with European Community guidelines.

Experimental design and diets

The rats were randomly divided into six groups: three control groups (C) and three Fe-deficient groups (ID) receiving the AIN-93G diet⁽¹²⁾ with a normal Fe level (45 mg Fe/kg diet) or with a low Fe level (5 mg Fe/kg diet)⁽¹³⁾, respectively, for 20, 30 or 40 d.

From the start of the study, the rats were maintained in an environment of controlled temperature (21–23°C), humidity (55%) and light–dark cycle (12 h–12 h), with *ad libitum* food and mineral-free water available. The last 7 d of the different periods of the study (from days 13 to 20, 23 to 30 and 33 to 40), records of weight, food intake, and urine and faeces were collected daily. On days 20, 30 or 40, the rats were fasted overnight and then anaesthetised by intraperitoneal injection of 5 mg sodium pentobarbital/100 g body weight (Sigma, St Louis, MO, USA). After median laparotomy, the rats were totally bled by cannulation of the abdominal aorta and aliquots with EDTA were analysed to measure the haematological parameters and the rest of the blood was centrifuged (1500 g, 4°C, 15 min) to measure thiobarbituric acid-reactive substances (TBARS) and superoxide dismutase (SOD) activity. The remaining blood was centrifuged without anticoagulant at 1500 g for 15 min at 4°C to separate the cells from the serum for subsequent analysis of Fe, ferritin and total Fe-binding capacity. The liver and brain (which were split in two portions, one to measure the Fe content and the rest to measure enzyme activity) were removed, washed with ice-cold saline solution (0.9%, w/v, NaCl) and stored immediately at –40°C until Fe analysis. Brain, liver and erythrocyte cytosolic fraction were prepared by differential centrifugation by hypotonic haemolysis according to the method described by Hanahan & Ekholm⁽¹⁴⁾, preserving the cytosol fraction at –80°C for further analyses. The final fractions were aliquoted, snap-frozen in liquid N₂ and stored at –80°C until analysis. Cytosolic protein contents were measured as described previously⁽¹⁵⁾. The activity of the antioxidant enzymes catalase (CAT) and glutathione peroxidase (GPx) in the brain, liver and erythrocyte cytosol was measured.

Analytical procedures

All the reagents were of analytical grade, and ultrapure water of 18 MΩ cm specific resistivity was obtained from a Milli-Q purification system (Millipore Corporation, Billerica, MA, USA).

DM

Moisture contents of the diet were determined by drying the materials in an oven at 105 ± 2°C until the weight remained constant (approximately 48 h).

Iron determination

Fe concentrations in the diets, faeces, urine, liver and brain were determined by atomic absorption spectrophotometry (Perkin–Elmer Analyst 1100B spectrometer with WinLab32 for AA software, Ueberlingen, Germany). The samples had been previously mineralised by the wet method in a sand bath (J.R. SELECTA, Barcelona, Spain). Samples of lyophilised bovine liver (certified reference material BCR 185; Community Bureau of References, Brussels, Belgium) were simultaneously used to check the Fe recovery (Fe value = 210 (SEM 6.0) mg/kg, mean (SEM) of five determinations, certified value 214 (SEM 5.0) mg/kg).

Haematological test

Erythrocytes, mean corpuscular volume, mean corpuscular Hb, platelets and Hb in blood samples were measured by using an automated haematology analyser (Sysmex KX-21, Tokyo, Japan). Serum ferritin concentration was determined using the Rat Ferritin ELISA Kit (Biovendor GmbH, Heidelberg, Germany). To calculate the rate of transferrin saturation, total Fe-binding capacity and serum Fe levels were determined colorimetrically and enzymatically using Sigma Diagnostics Iron and Total Iron-Binding Capacity reagents (Sigma Diagnostics). The rate of transferrin saturation was subsequently calculated using the following equation:

$$\begin{aligned} \text{Transferrin saturation (\%)} \\ &= \text{serum Fe concentration (\mu g/l) / total Fe} \\ &\quad \text{-binding capacity (\mu g/l)} \times 100. \end{aligned}$$

Thiobarbituric acid-reactive substance measurement

The extent of lipid peroxidation was evaluated in plasma by measuring the concentration of TBARS according to the methods described by Yagi⁽¹⁶⁾ and Ohkawa *et al.*⁽¹⁷⁾. Plasma (1 ml) was mixed with 1 ml of 15% TCA (Sigma-Aldrich) and centrifuged at 80 g for 10 min. Supernatant (1 ml) was mixed with 1 ml of tetrabutylammonium reagent (0.67%) and the mixture was kept in a boiling water-bath for 20 min. The reaction product was extracted and measured by spectrophotometric analysis (Thermo Spectronic, Rochester, NY, USA) at 532 nm. The assay procedure was calibrated using tetraethoxypropanone (Sigma-Aldrich) as a malodialdehyde source. The results were expressed as nmol TBARS per mg proteins.

Catalase activity

CAT activity was determined by following Aebi's method⁽¹⁸⁾, by monitoring at 240 nm the H₂O₂ decomposition, as a consequence of the catalytic activity of CAT. The activity was calculated from the first-order rate constant *K* (1/s).

Table 1. Haematological parameters in control (C) and iron-deficient (ID) rats*
(Mean values with their standard errors)

	Fe-deficiency effect (n 48)																
	Day 20 (n 16)					Day 30 (n 16)					Day 40 (n 16)					Time effect	
	C (n 8)		ID (n 8)		P	C (n 8)		ID (n 8)		P	C (n 8)		ID (n 8)		P	C (n 24)	ID (n 24)
	Mean	SEM	Mean	SEM		Mean	SEM	Mean	SEM		Mean	SEM	Mean	SEM		C (n 24)	ID (n 24)
Hb concentration (g/l)	123	6	99 ^A	3	0.001	116 ^a	7	85 ^B	4	0.0002	126	4	72 ^C	2	0.0001	NS	0.01
Haematocrit (%)	46.3	2.5	26.2	1.3	0.001	47.6	3.3	28.9	1.7	0.001	49.6	2.4	23.6	1.6	0.0001	NS	NS
Erythrocytes (10 ¹² /l)	6.5	0.4	5.3 ^{AB}	0.3	0.01	6.5	0.4	6.0 ^A	0.3	NS	7.7	0.3	5.1 ^B	0.2	0.001	NS	0.05
MCV (fl)	71.2	3.0	49.1	0.8	0.001	73.4	0.6	48.3	1.0	0.001	72.4	0.9	48.8	1.3	0.001	NS	NS
MCH (pg)	18.9	0.3	17.6 ^A	0.2	0.001	18.1	0.4	14.7 ^B	0.2	0.001	18.5	0.4	14.0 ^B	0.2	0.001	NS	0.001
Platelets (10 ⁹ /l)	703 ^a	66	1224 ^C	25	0.001	694 ^a	60	1336 ^B	17	0.0006	688 ^a	39	2026 ^A	13	0.0001	NS	0.0001
Serum Fe (µg/l)	1300 ^a	103	145	8	0.0001	903 ^b	95	164	10	0.0004	1200 ^a	99	181	18	0.0002	0.01	NS
TIBC (µg/l)	4560 ^a	110	7450 ^C	104	0.001	3840 ^b	151	10780 ^B	204	0.0003	3250 ^c	98	14500 ^A	305	0.0001	0.001	0.0001
Transferrin saturation (%)	29.3 ^b	1.0	2.0 ^A	0.01	0.0002	23.5 ^c	1.0	1.6 ^B	0.01	0.0004	40.3 ^a	2.9	1.4 ^C	0.01	0.0001	0.01	0.001
Serum ferritin (µg/l)	95.7 ^a	6.3	25.5 ^A	2.5	0.0003	78.2 ^b	3.7	23.4 ^A	1.1	0.0006	80.2 ^b	2.4	19.3 ^B	1.7	0.0001	0.01	0.05

MCV, mean corpuscular volume; MCH, mean corpuscular Hb; TIBC, total Fe-binding capacity.

^{a,b,c} For the time effect, the mean values of C groups within a row with unlike superscript letters were significantly different ($P \leq 0.05$).

^{A,B,C} For the time effect, the mean values of ID groups within a row with unlike superscript letters were significantly different ($P \leq 0.05$).

* As there were no interactions between Fe deficiency (C or ID) and time (20, 30 or 40), but there were significant main effects of Fe deficiency and significant main effects of time, the data were pooled to show pooled means for the main effects only.

Superoxide dismutase activity

SOD activity was determined according to the method described by Crapo *et al.*⁽¹⁹⁾. It was based on the inhibition by SOD in the reduction of cytochrome *c*, measured by spectrophotometry at 550 nm. One unit of SOD activity is defined as the amount of enzyme required to produce 50% inhibition of the rate of reduction of cytochrome *c*.

Glutathione peroxidase activity assay

GPx activity was measured using the technique of Flohé & Gunzler⁽²⁰⁾. This method is based on the instantaneous formation of oxidised glutathione during the reaction catalysed by GPx. This oxidised glutathione is continually reduced by an excess of glutathione reductase and NADPH present in the cuvette. The subsequent oxidation of NADPH to NADP⁺ was monitored spectrophotometrically at 340 nm. During the reaction, cumene hydroperoxide was used as a substrate.

Biological indices

The following indices were calculated from the data on Fe intake and faecal and urinary Fe excretion:

$$\text{Apparent digestibility coefficient (ADC)} = (\text{intake} - \text{faecal excretion}) \times 100 / \text{intake}; \text{Balance} = (\text{intake} - \text{faecal excretion}) - \text{urinary excretion}; R/I (\%) = \text{balance} \times 100 / \text{intake},$$

where *R* is the retention and *I* is the intake.

Statistical methods

Statistical analyses were performed using the SPSS computer program (SPSS, version 15.0, 2008; SPSS, Inc., Chicago, IL, USA). Differences between groups C *v.* ID were tested for statistical significance with Student's *t* test. Variance analysis by one-way methods was used to compare the different periods (20, 30 or 40 d) in the two groups of animals C and ID. Individual means were tested using pair-wise comparison with Tukey's multiple comparison test when main effects and interactions were significant. Data were analysed statistically by two-way ANOVA to determine the effects of Fe deficiency, the time (20, 30 or 40 d) and their interactions (Fe deficiency × time). The level of significance was set at *P* < 0.05. All the data are reported as mean values with their standard errors.

Results

The consumption of the Fe-restrictive diet provoked a decrease in Hb and haematocrit in all periods studied

Table 2. Weight change and iron nutritive utilisation in control (C) and iron-deficient (ID) rats* (Mean values with their standard errors)

	Fe-deficiency effect (n 48)																
	Day 20 (n 16)				Day 30 (n 16)				Day 40 (n 16)								
	C (n 8)		ID (n 8)		C (n 8)		ID (n 8)		C (n 8)		ID (n 8)						
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM					
Weight change (g/d)	3.2 ^b	0.2	3.1	0.2	NS	5.2 ^a	0.3	3.3	0.3	0.01	5.4 ^a	0.2	2.6	0.3	0.001	0.01	NS
Fe intake (µg/d)	669 ^b	26	67 ^b	1	0.0001	792 ^a	35	77 ^a	4	0.0001	766 ^a	26	75 ^a	2	0.0001	0.02	0.01
Faecal Fe (µg/d)	491 ^b	34	46 ^b	0.6	0.0002	563 ^a	21	50 ^a	1	0.0002	557 ^a	7	46 ^b	1	0.0001	0.05	0.01
ADC (%)	27	3	32 ^b	2	NS	29	1	35 ^{a,b}	2	0.01	27	3	38 ^a	2	0.001	NS	0.05
Urinary Fe (µg/d)	2.6	0.4	1.1 ^b	0.1	0.001	3.3	0.2	1.5 ^a	0.2	0.002	2.7	0.2	1.1 ^b	0.1	0.001	NS	0.001
R/I (%)	27	3	30 ^b	2	NS	28	1	33 ^{a,b}	2	0.05	26	3	36 ^a	2	0.01	NS	0.01

ADC, apparent digestibility coefficient; R/I, retention/intake.

^{a,b} For time effect, the mean values of C groups within a row with unlike superscript letters were significantly different (*P* ≤ 0.05).

^{A,B} For time effect, the mean values of ID groups within a row with unlike superscript letters were significantly different (*P* ≤ 0.05).

* As there were no interactions between Fe deficiency (C or ID) and time (20, 30 or 40), but there were significant main effects of Fe deficiency and significant main effects of time, the data were pooled to show pooled means for the main effects only.

(significant level, Table 1), especially the decrease in haematocrit at day 40, diminishing by 52% with regard to their counterparts C ($P=0.0001$). The Hb decrease became more pronounced in the ID group as the degree of Fe deficiency advanced, being significant when comparing the periods 30 *v.* 40 ($P=0.048$) and 20 *v.* 40 ($P=0.0012$), whereas the levels of haematocrit were kept low in the three studied periods, without significant differences between them. Likewise, the nutritional Fe deficiency diminished the erythrocytes in the three periods, being significant at day 20 ($P=0.009$) and 40 ($P=0.0011$), with regard to C groups. In addition, in the course of the development of the Fe deficiency, the lowest erythrocyte count corresponds to day 40 compared with day 30 ($P=0.039$). In relation to the erythrocytic indexes mean corpuscular volume and mean corpuscular Hb, both diminished due to Fe deficiency ($P=0.001$) during the three periods studied. In ID rats, the number of platelets increased in the three studied periods (significant level, Table 1), reaching values double that of the initial count at days 20 and 30 of supplying the Fe-restrictive diet and treble in day 40, with regard to the animals that received a diet with normal Fe content. As the Fe deficiency was established, there was an increase in the number of platelets, reaching values of 2026 (SEM 13) at day 40 *v.* 1224 (SEM 25) at day 20 ($P=0.0002$). The reduced serum Fe of the ID group at day 20 (145 (SEM 8) $\mu\text{g/l}$) compared with that of the C group at the same time point (1300 (SEM 103) $\mu\text{g/l}$) ($P<0.0001$) is also noteworthy, values that remained low during the whole development of the Fe deficiency. In relation to the percentage saturation of transferrin, the drastic decrease in ID rats, which decreased further in the course of the development of the Fe deficiency (significant level, Table 1) is noteworthy. Percentage saturation of transferrin was 1.4 (SEM 0.01) at day 40 for the ID group, compared with 40.3 (SEM 2.9) for the C group ($P=0.0001$). With regard to the levels of serum ferritin, in ID rats these were about a quarter of those in the C rats in all three studied periods (significant level, Table 1).

Table 2 summarises the weight change and digestive and metabolic utilisation of Fe in both groups of animals. At the beginning of the experiment, the rats recently weaned with 21 d of age had a mean weight of 43.8 (SEM 3.5) g. On day 30 of the study, significant differences in the increase of weight between both groups of animals were observed, being lower in the ID group ($P=0.009$), differences that were accentuated after 40 d of supplying the Fe-restrictive diet, diminishing the increase of weight in ID animals to half that of the C rats ($P=0.0011$). After 20 d of consuming a diet with a low Fe content, ID rats featured slightly higher values of digestive utilisation (ADC) and retention (R/I) of this mineral with regard to their controls and the differences became higher as time of consumption of the Fe-restrictive diet increased: ADC ($P=0.01$ at day 30 and $P=0.0012$ at day 40) and R/I of Fe ($P=0.0047$ at day 30 and $P=0.011$ at day 40). In the course of the study, the

Table 3. Iron content in liver and brain of control (C) and iron-deficient (ID) rats* (Mean values with their standard errors)

	Fe-deficiency effect (n 48)																	
	Day 20 (n 16)			Day 30 (n 16)			Day 40 (n 16)			C (n 8)			ID (n 8)			Time effect		
	Mean	SEM	P	Mean	SEM	P	Mean	SEM	P	Mean	SEM	P	Mean	SEM	P	Mean	SEM	P
Liver ($\mu\text{g/g}$ dry weight)	423	65	0.0001	380	19	0.0001	114 ^B	7	0.0001	405	46	0.001	149 ^A	7	0.001	NS	NS	0.01
Brain ($\mu\text{g/g}$ dry weight)	121	12	0.0002	110	4	0.0002	37 ^B	2	0.001	123	12	0.01	53 ^A	3	0.01	NS	NS	0.01

^{A,B} For time effect, the mean values of ID groups within a row with unlike superscript letters were significantly different ($P \leq 0.05$).

* As there were no interactions between Fe deficiency (C or ID) and time (20, 30 or 40), but there were significant main effects of Fe deficiency and significant main effects of time, the data were pooled to show pooled means for the main effects only.

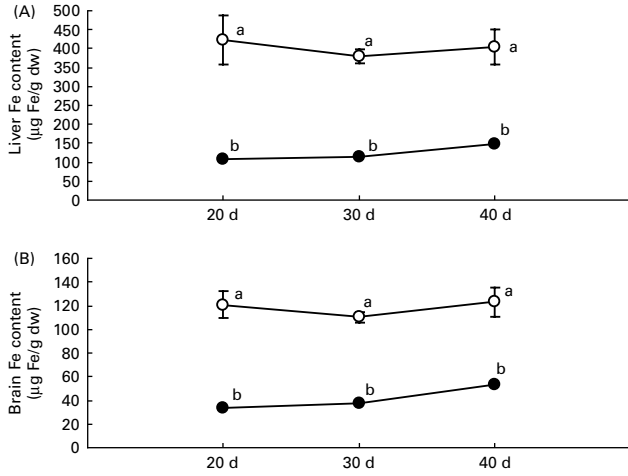


Fig. 1. Fe content in (A) liver and (B) brain over time (20, 30 or 40 d). ○, Control groups; ●, Fe-deficient groups. The means values with standard errors are represented by vertical bars. For details of groups, see Table 3. The data were analysed by two-way ANOVA with repeated measures. There was a significant main effect for time and for Fe deficiency. There were no significant main effects for Fe-deficiency × time interaction. ^{a,b} Mean values with unlike letters were significantly different ($P \leq 0.05$). dw, Dry weight.

nutritive utilisation (ADC and *R/D*) in C rats was not modified in any of the studied periods. Nevertheless, in ID rats, the nutritive utilisation of Fe increased progressively in all the periods, this difference being statistically significant when comparing day 40 with day 20 ($P = 0.009$ for ADC and $P = 0.0048$ for *R/D*).

Table 3 summarises Fe concentrations in the liver and brain. Fe deposits in both the organs of ID rats were almost four times lower than in the C group at day 20 of Fe deficiency, with values of 107 (SEM 8) *v.* 423 (SEM 65) µg Fe/g dry weight for liver (Fig. 1(A)) and 34 (SEM 2) *v.* 121 (SEM 12) µg Fe/g dry weight for brain (Fig. 1(B)). This lower Fe deposit remained at day 30 ($P = 0.0009$ for the C group and $P = 0.006$ for the ID group), but there was a slight recovery of the Fe content in the liver and brain at day 40 ($P = 0.005$) with regard to the previous periods (Fig. 1(A) and (B)).

Under the present experimental conditions, a decrease in SOD activities was observed in plasma ($P = 0.01$), together with CAT ($P = 0.001$) (Fig. 2(A)) and GPx ($P = 0.05$) (Fig. 2(B)) in hepatic cytosol of ID rats at day 20 of the study compared with the C rats, with no differences between the C and ID groups in the other studied periods (30 and 40 d). Nevertheless, neither lipid peroxidation (TBARS) nor the studied mechanisms of antioxidant defence of SOD in plasma and CAT (Fig. 2(C)) and GPx (Fig. 2(D)) in the cytosolic fraction of brain and erythrocytes were altered by the Fe deficiency in any of the studied periods (Table 4).

Discussion

At the beginning of the study, all the haematological parameters studied were within the normal limits described

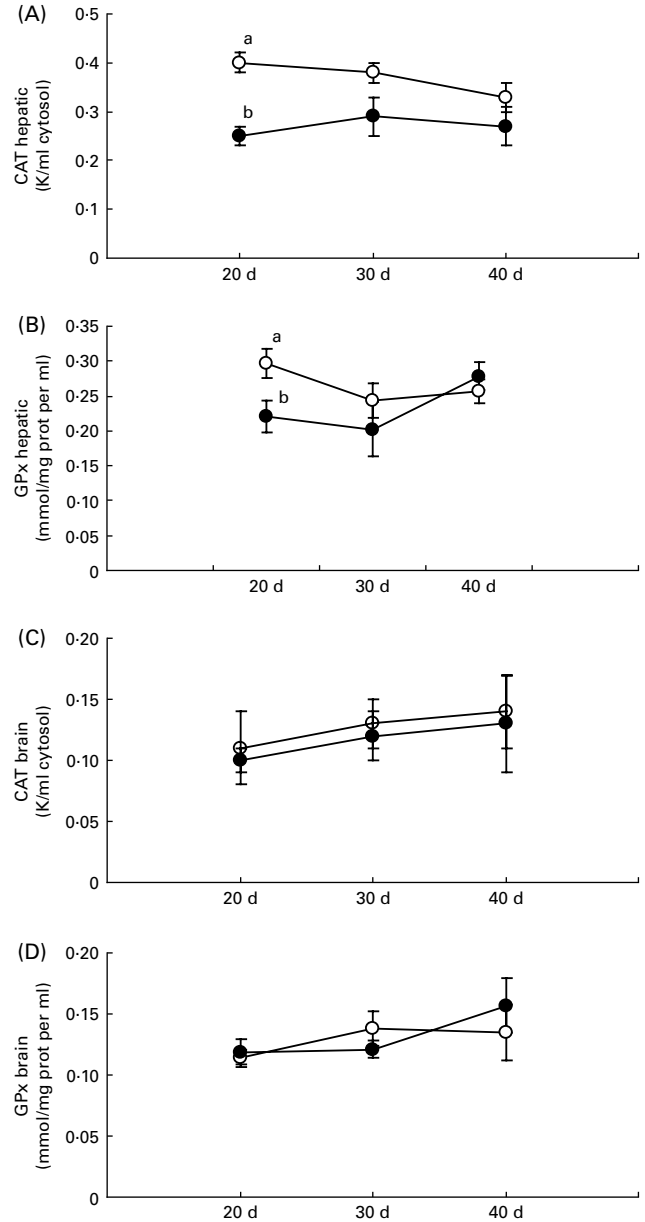


Fig. 2. Catalase (CAT) and glutathione peroxidase (GPx) activity in liver ((A) and (B)) and brain ((C) and (D)) over time (20, 30 or 40 d). ○, Control groups; ●, Fe-deficient groups. The means values with their standard errors are represented by vertical bars. For details of groups, see Table 4. The data were analysed by two-way ANOVA with repeated measures. There was a significant main effect for Fe deficiency. There were no significant main effects for time or Fe deficiency × time interaction. ^{a,b} Mean values with unlike letters were significantly different ($P \leq 0.05$). prot, Protein.

in the literature for this species at this age⁽²¹⁾. At day 20, the haematological parameters of the C rats were within normal limits, except for serum Fe, as a consequence of the needs imposed by growth⁽²²⁾ and of the increase of the demands of Fe for the synthesis of Fe-dependent enzymes⁽²³⁾. In ID rats, Hb, erythrocytes, haematocrit, mean corpuscular volume, mean corpuscular Hb percentage saturation of transferrin and serum ferritin decreased in a marked way, whereas the platelets were higher. Fe deficiency is classified in three stages according to severity:

Table 4. Antioxidant defence and thiobarbituric acid-reactive substances (TBARS) production in control (C) and iron-deficient (ID) rats* (Mean values with their standard errors)

	Fe-deficiency effect (n 48)											
	Day 20 (n 16)				Day 30 (n 16)				Day 40 (n 16)			
	C (n 8)		ID (n 8)		C (n 8)		ID (n 8)		C (n 8)		ID (n 8)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
SOD plasma (U/mg protein)	2.49	0.08	2.14**	0.10	2.35	0.25	2.02	0.06	2.62	0.23	2.36	0.25
CAT hepatic (K/ml cytosol)	0.40	0.02	0.25***	0.02	0.38	0.02	0.29	0.04	0.33	0.03	0.27	0.04
CAT brain (K/ml cytosol)	0.11	0.03	0.10	0.01	0.13	0.02	0.12	0.02	0.14	0.03	0.13	0.04
CAT erythrocyte (K/ml cytosol)	0.21	0.03	0.23	0.02	0.20	0.03	0.21	0.04	0.21	0.06	0.20	0.03
GPx hepatic (mmol/mg protein per ml)	0.30	0.02	0.22*	0.02	0.24	0.03	0.20	0.04	0.26	0.02	0.28	0.02
GPx brain (mmol/mg protein per ml)	0.11	0.01	0.12	0.01	0.14	0.01	0.12	0.01	0.14	0.02	0.16	0.02
GPx erythrocyte (mmol/mg protein per ml)	0.26	0.02	0.30	0.02	0.30	0.04	0.32	0.03	0.26	0.01	0.30	0.03
TBARS (nmol/mg protein)	3.92	0.35	4.39	0.34	4.02	0.43	4.00	0.15	4.32	0.58	4.40	0.49

SOD, superoxide dismutase activity; CAT, catalase activity; GPx, glutathione peroxidase activity.

Mean value was significantly different from that of the C group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

* For the time effect, the data were analysed by one-way ANOVA, but there were no significant main effects.

depletion, marginal deficiency and anaemia. Depletion is characterised by depleted Fe stores with normal Fe-dependent protein production and normal Hb concentrations. Marginal deficiency is characterised by depleted Fe stores, reduced Fe-dependent protein production (e.g. oxidative enzymes) and normal Hb concentrations. Anaemia, the most severe form of deficiency, is characterised by depleted Fe stores, reduced Hb concentrations and reduced Fe-dependent oxidative enzyme concentrations⁽²³⁾. The drastic decrease of serum Fe, saturation of transferrin and serum ferritin, at day 20 can be owed not only to the needs imposed by growth but also to the consumption of a Fe-restrictive diet. The critical period in the development of nutritional Fe-deficiency anaemia happened between days 30 and 40, where there was a notable reduction in the weight gain and Hb concentration, together with a marked increase in the platelets, parameters that are indicative of the development of severe anaemia. The marked decrease of Hb levels and the other haematological parameters studied is due to the fact that the organism is unable to obtain Fe from body stores without compromising Fe-dependent enzymic mechanisms^(23,24).

The average initial weight of the rats was within the normal margins described in the literature of this species^(21,25). The results of the present study have demonstrated that a parallelism exists at day 20 in the weight gain of both the groups of animals, C and ID, which indicates that Fe stored during the fetal period and during lactation is enough to cover the animals' needs in their early life stage. Because at day 40, the concentration of Hb, erythrocyte count and haematocrit were significantly reduced in ID animals, the supply of oxygen to the cells was lower, which had repercussions in a minor weight gain of this animal group. This is in agreement with the results found by Schneider *et al.*⁽²⁶⁾, reporting that in children 12–36 months old, it is possible that low Hb concentrations

limited the rate of weight gain, which would explain the association observed.

The nutritive utilisation of Fe found in ID rats increases progressively as Fe deficiency advances, and this can be due to the fact that this pathology produces a depletion of the body Fe stored⁽²³⁾, which favours the uptake by simple diffusion following the gradient of concentration of this mineral⁽²⁷⁾. In addition, in situations of Fe deficiency, the synthesis of divalent metal transporter 1 is increased⁽²⁸⁾ as well as the expression of ferroportin 1⁽²⁹⁾. Therefore, these receptors favour the intestinal absorption of Fe in situations of deficiency of this mineral⁽³⁰⁾.

According to Brownlie *et al.*⁽²³⁾, Fe deficiency is characterised by depletion of Fe stores; however, in the present study, there was a slight recovery in the Fe content of the liver and brain at day 40 of the Fe deficiency in spite of the fact that these levels continue being two times below those found in C rats; this indicates the importance of this mineral during early life in both organs. The first years of life are critical for neurological development; the pace of neurological development in young children aged 0–4 years is rapid, including critical periods of neural circuit formation and myelination in the brain. Fe in oligodendrocytes is required for proper myelination of the neurons used in the sensory system and learning and interacting behaviours⁽¹⁰⁾.

There is a controversy about the susceptibility of cells to lipid peroxidation in Fe deficiency: some authors argue that there is no difference in lipid peroxidation in ID patients compared to control ones^(31,32). Alexander *et al.*⁽⁶⁾ showed that reduced dietary Fe is considered to be beneficial in improving acute and chronic liver injuries by reducing oxidative stress. Other authors reported that while oxidants are increased, antioxidants are decreased and as a result, the oxidative/antioxidative balance shifted towards the oxidative side in patients with Fe deficiency^(33,34). Moreover, there is certain ambiguity in antioxidant enzyme status:

some authors reported that antioxidant enzymes were low⁽³⁵⁾ and others stated that antioxidant enzymes were increased in Fe-deficiency anaemia⁽³⁶⁾. In the present study, as the levels of serum Fe were drastically diminished at day 20, keeping these low levels during the progression of anaemia, it is logical to think once again that the decrease in serum Fe is translated into a minor production of reactive oxygen species, which might explain the decrease in SOD in the plasma compartment at day 20. TBARS production was similar in C and ID rats, suggesting once again that the total antioxidant capacity of the organism is adapted in the course of the development of anaemia, due to the adequate activities of the antioxidant enzymes CAT, GPx and SOD that are involved in the reduction of the peroxides, which can attack PUFA, therefore avoiding lipid peroxidation and the subsequent formation of TBARS. Rats at birth have a high level of serum Fe, but during the first weeks of life many erythrocytes haemolyse, in a physiological process to normalise the high erythrocyte count featured at this point. The Fe that is liberated is stored principally in the liver and the spleen. Since the mother's milk is a poor source of Fe, this is used during the first days of life for helping to synthesise Hb and increase the number of erythrocytes, a fact that becomes extremely important as the animal grows, to satisfy the demands of oxygen and oxidative metabolism. Fe stored in the newborn animal together with the low amount that is supplied from the mother's milk is sufficient for a short period of time, but from this moment it is necessary that the diet provides a suitable level of Fe^(37,38). In the present study, because Fe deficiency had been induced in growing rats, the excess of serum Fe which the animals had at birth was not stored, but was used in an unsuccessful attempt to replete body Fe, trying to maintain the serum levels within physiological range. Due to the fact that the ID animals had a minor content of hepatic Fe in all the periods studied compared with the C animals, especially at day 20 of the Fe deficiency, this can be the reason why a decrease was observed in the activities of CAT and GPx, since Fe deficiency would exert a protective effect avoiding the generation of reactive oxygen species (especially hydroxyl radicals via Fenton and Haber-Weiss chemistry)⁽³⁹⁾. Nevertheless, in spite of the low Fe stores in the brain of ID rats, two to three times lower compared with those in the C rats, especially at day 20, changes were not observed in the activity of the antioxidant enzymes CAT and GPx by Fe deficiency in any of the periods studied. CAT is a ferric haeme-containing enzyme. Fe deficiency can reduce the activity of Fe-dependent enzymes⁽⁴⁰⁾; however, under our experimental conditions, CAT activity in the brain cytosolic fraction among ID and C rats did not differ.

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