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Effects of marginal iron overload on iron homeostasis and immune function in alveolar macrophages isolated from pregnant and normal rats

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Abstract The effects of changes in macrophage iron status, induced by single or multiple iron injections, iron depletion or pregnancy, on both immune function and mRNA expression of genes involved in iron influx and egress have been evaluated. Macrophages isolated from iron deficient rats, or pregnant rats at day 21 of gestation, either supplemented with a single dose of iron dextran, 10 mg, at the commencement of pregnancy, or not, showed significant increases of macrophage *ferroportin* mRNA expression, which was paralleled by significant decreases in hepatic *Hamp* mRNA expression. IRP activity in macrophages was not significantly altered by iron status or the inducement of pregnancy ± a single iron supplement. Macrophage immune function was significantly altered by iron supplementation and pregnancy. Iron supplementation, alone or combined with pregnancy, increased the activities of both NADPH oxidase and nuclear factor kappa B (NFκB). In contrast, the imposition of pregnancy reduced the ability of these parameters to respond to an inflammatory stimuli. Increasing iron status, if only marginally, will reduce the ability of macrophages to mount a sustained response to inflammation as well as altering iron homeostatic mechanisms.

Keywords Iron homeostasis · Pregnancy · Macrophages · NFκB · Nitric oxide synthase

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Abbreviations

NO	Nitric oxide
DcytB	Duodenal cytochrome B
DMEM	Dubecco's modified eagle medium
DMT1	Divalent metal-ion transporter 1
EDTA	Ethylenediamine tetra acetate
fmlp	N formyl methionyl leucyl phenylalanine
HEPES	N-2-hydroxylethylpiperazine
ICP-AES	Inductively coupled plasma-atomic emission spectroscopy
IRP	Iron regulatory protein
IRE	Iron regulatory element
IFNγ	Interferon gamma
LNMA	N^G-methyl-L-arginine

LPS	Lipopolysaccharide
NFκB	Nuclear factor kappa B
NOS	Nitric oxide synthase
PMA	Phorbol-12 myristate-13 acetate
RT-PCR	Reverse transcriptase polymerase chain reaction
TIBC	Total iron binding capacity
TfR1	Transferrin receptor 1
TNFα	Tumor necrosis factor alpha

Introduction

It is now common practice to supplement various foodstuffs with small amounts of iron, in the belief that this could be beneficial to health. Furthermore, in pregnancy, different iron supplementation regimes are now adopted; either for all subjects, regardless of iron status (Stolzfus and Dreyfuss 1988; WHO 2001), or only for iron deficient subjects (Rioux and LeBlanc 2007), who receive iron supplements during pregnancy. Therefore marginal iron overload could occur if there is prolonged intake of iron-fortified food or iron supplements before or during pregnancy. This may have adverse effects on normal cellular function.

Enterocytes and macrophages play an important role in both iron uptake and efflux (as a function of iron surfeit and depletion), by regulating the expression of a number of genes involved in iron homeostasis. In addition, to this transcriptional control, regulation at the level of post-translation of pre-existing mRNAs into protein by the ribosomal protein synthesising machinery through the IRP-IRE system may further 'fine tune' this process. It is thought that the IRE/IRP system plays an important regulatory role in the gut and liver (Millard et al. 2004), although its precise function in macrophages remains unclear. Alterations in the expression of specific mRNAs involved in iron homeostasis induced by the imposition of pregnancy as well as an elevated iron status at the commencement of pregnancy have not previously been investigated.

Iron homeostasis is an important factor in immune function; iron deficiency is associated with reversible abnormalities of immune function, while iron supplementation has been associated with acute exacerbation of infection (Oppenheimer 2001). In our previous studies (Crichton et al. 2002; Legssyer et al. 1999, 2003; Ward et al. 2004) we have shown that macrophage function is altered by changes in iron homeostasis. Since pregnancy is a condition of oxidative stress, arising from increased placental mitochondrial activity and production of reactive oxygen species (Myatt and Cui 2004), any excess iron, particularly within the macrophages, could lead to an increased oxidative burden, and an elevation of the inflammatory response.

In the present studies, we wished to investigate the effects of an increased iron status during pregnancy on both immune function, e.g. NADPH oxidase and Nuclear factor κB (NFκB), and mRNA expression of various genes involved in iron homeostasis, in alveolar macrophages.

Materials and methods

Animal experiments

Female Wistar rats, 200–250 g were housed in polypropylene cages (*Sources*: *Iffa Credo*, *Belgium*, *Breeding Colony Unite de Biologie*, *LLN*) and allowed free access to normal diet and water ad libitum in polypropylene bottles with stainless steel conical tops. All animal procedures were in strict accordance with the recommendations of EEC (86/609/CEF) and with the Belgian 'projet de loi' (Moniteur Belge 19.02.1992, p. 3437) on the care and use of laboratory animals.

The rats were mated overnight and pregnancy confirmed by the presence of sperm in the vagina. A single dose of iron, 10 mg, (50 mg/kg) was administered as iron dextran (*Vifor Pharmaceuticals, St. Gallen, Switzerland*) intramuscularly on day 1 of pregnancy while the remaining pregnant rats received a sham injection of saline. A third group of non-pregnant rats received a single intramuscular dose, 10 mg, of iron dextran.

A further two groups of rats, which were either iron overloaded or iron depleted, were included as reference groups to ascertain the response of various immune factors to an increased or decreased iron burden. Non-pregnant female rats, 50–60 g at the commencement of the study, were injected intraperitoneally 3×/week for 4 weeks with iron dextran (*Vifor Pharmaceuticals Inc, St. Gallen, Switzerland*) 10.4 mg/dose, total dose = 125 mg iron. At the conclusion of the iron supplementation regime, the weight of the rats was 250 ± 10 g. Iron deficiency

was induced in a fifth group of non-pregnant female rats, 50–60 g at the commencement of the study, by the administration of an iron deficient diet for four weeks (*Carfil Quality, OUD-Turhoud Belgium*; Iron content $\leq$ 0.001 ppm). The mean weight of these rats at the end of 4 weeks was 213 $\pm$ 5 g.

At the end of 4 weeks of high iron loading or iron depletion experiments, or day 21 of pregnancy $\pm$ iron supplementation, or 21 days after a single intramuscular injection of iron, the rats were anesthetised with Nembutal, 0.5 g/kg. The alveolar macrophages were removed by repeated pulmonary lavage with 50 ml phosphate buffered saline. The cells were recovered after centrifugation at 1,200 rpm for 10 min. A gut scraping was prepared from the first centimetre of the duodenum, and a small portion of liver was collected from each animal and snap frozen at −80°C in liquid nitrogen. Blood was collected by cardiac puncture for the analyses of haematological parameters (*Labatoire medical du Sud, Namur, Belgium*).

Hepatic and alveolar macrophage iron content

An aliquot of alveolar macrophages, 5×10^5 cells, or of liver, 100 mg, was homogenised in water, prior to overnight digestion with 1.5 ml concentrated nitric acid (*Suprar grade*). Water, 1.2 ml, was added and the sample analysed for its iron content by inductively coupled plasma emission, ICP-AES. The protein concentration in each of the macrophage preparations was analysed by the method of Bradford (1976) (*Biorad, Belgium*), and macrophage iron results expressed as μg Fe/mg protein, while liver iron is expressed as μg Fe/g wet weight.

mRNA expression of genes involved in iron homeostasis

Semi-quantitative analysis of mRNA, RT-PCR, in alveolar macrophages and gut

Gut scrapings from the duodenum, (approximately 100 μg) were thawed and homogenised in Trizol, (*Invitrogen*), according to the manufacturer's instructions. Alveolar macrophages were isolated from three rats, pooled, centrifuged at 1,200 rpm, and a minimum of 5×10^6 cell immediately suspended in Trizol. A minimum of 12 rats, i.e. four analyses, was used in each group for the gene expression experiments. After isolation of total RNA, its purity was assessed by both agarose gel electrophoresis and spectroscopic measurement at 260 and 280 nm to ensure a ratio between 1.6 and 1.8. The housekeeping gene actin was used for normalisation. The oligonucleotide primer sequences together with the annealing temperature used for the amplification of each of the genes are shown in Table 1a.

Enterocyte and hepatic mRNA expression by real time PCR

RNA was extracted from enterocytes and liver portions using Trizol reagent (*Invitrogen*) according to the manufacturer's instructions. Freshly prepared RNA (1 μg) was reverse transcribed using a cDNA reverse transcription kit (*Promega*, according to the manufacturer's instruction). All reactions were performed on a real time PCR machine (*Lightcycler, Roche*) using β-actin as an internal standard. Each reaction was performed in duplicate and contained 10 pmoles of specific primers (as shown in Table 1a and b), 1× Sybr Green Mastermix (*QIagen*), and 1.0 μl of cDNA in a 25 μl reaction. Samples without cDNA were included as negative controls. Cycle threshold (Ct) values were obtained for each gene of interest and the β-actin internal standard. Gene expression was normalised to β-actin and represented as ΔCt values. For each sample the mean of the ΔCt values was calculated. Relative gene expression was normalised to 1.0 (100%) of controls. Statistical analysis was by one-way ANOVA.

Preparation of alveolar macrophages for IRP analyses

Macrophages were isolated from three rats in each group and pooled (approximately 5×10^6 cells). A minimum of 12 rats were used for each treatment group (four analyses in each treatment group). The cells were recovered after centrifugation at 1,200 rpm and lysed in 8.3 μl/10^6 cells of Munro buffer, (10 mM Hepes pH 7.6, 3 mM $MgCl_2$, 40 mM KCl, 5% glycerol, 1 mM DTT), supplemented with 0.2% NP-40. The plasmid pST-fer, containing an oligonucleotide corresponding to bases 31–58 of the 5′ untranslated region of human ferritin heavy chain mRNA (kindly provided by Professor L. Kühn) was amplified and prepared as previously published (Ward et al. 1994). Aliquots of the cytosolic fractions of liver (10 μg protein), and macrophage

Table 1a Methodology for amplification of mRNA expression by reverse transcriptase polymerase chain reaction RT-PCR

Denaturation 1 min at 94°C		
Hybridisation—1 min at appropriate temperature		
	Cycles	°C
DcytB (*CYBRD1*)		
Sense: 5′-GCAGCGGGCTCGAGTTTA-3′	35	59
Antisense: 5′-TTCCAGGTCCATGGCAGTCT-3′		
DMT1 (*Slc11A2*)		
Sense: 5′-GGCTTTCTTATGAGCTTGCCTA-3′	34	54
Antisense: 5′-GGAGCACCCAGAGCAGCTTA-3′		
Ferroportin (*Slc40A1*)		
Sense: 5′-TTGCAGGAGTCATTGCTGCTA-3′	27	50
Antisense: 5′-TGGAGTTCTGCACACCATTGAT-3′		
TfR1		
Sense: 5′-CCTCGTGAGGCTGGATCTCAA-3′	30	59
Antisense: 5′-TGGACCAGTTTACCAGTAACTT-3′		
Actin		
Sense: 5′-GACGTTGACATCCGTAAAG-3′	27	59
Antisense: 5′-CAGTAACAGTCCGCCT-3′		
Elongation 1 min at 72°C		
Analysis by electrophoresis		

Table 1b Methodology for amplification of mRNA expression by real time polymerase chain reaction

Hamp
Forward: CACGAGGGCAGGACAGAAGGCAAG
Reverse: CAAGGTCATTGCTGGGGTAGGACAG

(20 μg protein) samples were incubated with the T7 transcribed RNA in the presence or absence of 2% β-mercaptoethanol. After incubation for 30 min, 1 unit RNAase T1 was added, incubated for 10 min after which heparin, 5 mg/ml, was added and left for 10 min. Loading buffer, 30 mM Tris–HCl pH 7.5, 40% sucrose, 0.2% bromophenol blue. Bromophenol Blue was added to the samples prior to their loading onto gels. Polyacrylamide gels, 6% were prepared with 0.3× TBE as the running buffer and electrophoresis was at 150 V for 3 h at 4°C. The gels were then dried, exposed for autoradiography and quantitated.

NADPH oxidase activity

Measurement of NADPH oxidase in macrophages was by chemiluminescence, with the Abel Cell Activation Test Kit. Macrophages, 2×10^5 or 4×10^5 cells, were suspended in the luminescence solution and baseline was measured in a thermostatically controlled luminometer at 37°C. The respiratory burst was then stimulated with either N-formyl-methionyl-leucyl-phenylalanine peptide, *fmlp*, 25–100 ng/ml, or phorbol-12 myristate-13 acetate, PMA, 25–100 ng/ml. The response was recorded over a period of 20 min. The area under the curve was calculated.

NF-κB analysis

Macrophage nuclear extracts were prepared from each treatment group, (5×10^6 cells pooled from three rats), n = 12 rats, before and after stimulation with LPS, 1 μg/ml, +TNFα 4 U/ml, in vitro for 30 min. Cells were prepared as previously described for band shift assay (Ward et al. 1996). The protein concentrations were assayed by BioRad in one of the aliquots prior to the electrophoretic mobility shift assay.

The nuclear fraction containing 20 μg protein was incubated for 30 min at room temperature with 0.2 ng ^{32}P-labelled oligonucleotide probe, 5′-GATCAGGG ACTTTCCGCTGGGGACTTTCCAG-3′, (sequenced and labelled by the method described by Kustermans et al. 2005) 1 mg BSA and 1.25 mg poly(dI-dC), poly (dI-dC) (*Pharmacia Biotech Benelux*) in buffer (20 mM HEPES/KOH, 75 mM NaCl, 1 mM EDTA, 5%

(by volume) glycerol, 0.5 mM $MgCl_2$, 1 mM DTT, pH 7.9) in a final volume 20 µl.

DNA–protein complexes were resolved on a non-denaturing 6% (w/v) polyacrylamide gel run for 4 h at 180 V in buffer (2.5 mM Tris, 2.5 mM H_3BO_3, 2 mM EDTA, pH 8.5). The gel was then dried and autoradiographed on a Fuji X-ray film (*General Electrics, Antwerp, Belgium*). For competition experiments, unlabelled probe, wild type, 5′-GATCAGGGACT TTCCGCTGGGGACTTTCCAG-3′ or mutated 5′-GA TCACTCACTTTCCGCTGCTCACTTTCCAG-3′ was added in excess (50×) in buffer. In each experiment the band of the DNA–protein complex from the unstimulated and stimulated macrophages was verified by comparison to the band obtained after stimulation of U937 with LPS, 10 µg/ml. U937 cells are a human promyeloid call line (gift of Prof. J. Piette), which were cultured in RPMI 1640 containing 2 mM glutamine and 10% foetal calf serum.

NO release from alveolar macrophages after in vitro stimulation

Alveolar macrophages, 10×10^5 cells or 20×10^5 cells, were plated on plastic wells in Dulbeco's modified eagle medium (DMEM) containing 10% foetal calf serum, 100 µg/ml streptomycin, 100 U/ml penicillin at 37°C under 5% CO_2. Cell viability was measured by trypan blue uptake extrusion (>98%). The cells were stimulated in medium containing lipopolysaccharide (LPS) 1 µg/ml ± interferon gamma (IFNγ), 500 U/ml, for 44 h. Nitric oxide, NO, released from the cultured alveolar macrophages, was assayed by the accumulation of the stable end product nitrite in the media. A 100 µl sample was removed from the incubation media after 44 h incubation, and incubated with an equal volume of Griess reagent (1% sulphanilamide; 0.1% N-1 naphthyethylenediamine dihydrochloride; 2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was read on a microplate reader. Nitrite standards in the appropriate reference range (0–50 µM) were prepared from sodium nitrite.

SOD activity in macrophages

Superoxide dismutase was analysed by the method of Beauchamp and Fridovich (1971). In this method, xanthine and xanthine oxidase produce superoxide in vitro, which will reduce the chromagen nitroblue tetrazolium (NTBB). The SOD in the sample competes with the NTBB for the superoxide, such that the activity of SOD is inversely proportional to the intensity of colour produced. One unit of SOD corresponds to an inhibition of 50% in the reduction of NTBB by superoxide.

Statistical analyses

All of the results are presented as mean ± standard deviation. Statistical analysis was calculated by one way ANOVA with significance verified by Fisher '*t*' test, and set at <0.05.

Results

Haematological parameters

The values for haemoglobin, serum iron, total iron binding capacity and transferrin saturation in the different pregnant groups, at day 14 and 21 gestation, are shown in Table 2. As the pregnancy reached term, day 21, the foetal demand for iron increased as reflected by significant reductions in both haemoglobin concentration and total iron binding capacity in both pregnant groups of rats regardless of iron supplementation. However, transferrin saturation and serum iron decreased significantly only in the non-supplemented pregnant group at day 21 gestation.

Total hepatic iron content

Hepatic iron content had declined significantly, by approximately 4-fold, in the non-supplemented animals on day 21 gestation (Table 3). The pregnant rats that had received a single iron supplement on day 1 gestation, also showed a significant decrease in total hepatic iron on day 21 gestation although this was only by 50% compared to controls. No significant alterations in total hepatic zinc and copper content were assayed in either pregnant group (Table 3).

Macrophage iron content

Macrophages isolated from the pregnant rats, whether supplemented on day 1 with iron or not, showed similar elevated levels of iron, 0.57 ± 0.03 and

Table 2 Haematological parameters in pregnant rats on day 21 gestation ± single iron supplement on day 1 gestation

	Haemoglobin (g/dl)	Serum iron (g%)	TIBC (g%)	Transferrin saturation (%)
Controls (n = 22)	12.2 ± 1.0	360 ± 92	526 ± 41	69 ± 16
Pregnant (n = 12)				
14 days	10.9 ± 0.9*	347 ± 104	503 ± 75	68 ± 14
21 days	9.7 ± 0.8*	183 ± 85*	372 ± 43*	49 ± 23*
Pregnant + single iron supplement, 10 mg (n = 12)				
14 days	9.0 ± 1.4*	271 ± 4.2	413 ± 77	59 ± 11
21 days	9.8 ± 0.9*	254 ± 46	392 ± 58*	58 ± 4
Control + single iron supplement, 10 mg (n = 12)				
14 days	11.9 ± 1.1	339 ± 101	506 ± 60	68 ± 12.5
21 days	13.1 ± 0.5	383 ± 50	500 ± 49	77 ± 9

Results are expressed as mean ± standard deviation

Significance * $P \leq 0.05$

Table 3 Total maternal hepatic copper, iron and zinc content of pregnant rats on day 14 and day 21 gestation after single iron supplement (10 mg) on day 1 of pregnancy

	Copper (μg/g liver)	Iron (μg/g liver)	Zinc (μg/g liver)
Control (n = 22)	5.7 ± 1	349 ± 68	39 ± 5
Pregnant (n = 12)			
14 days	4.8 ± 2	191 ± 10*	42 ± 45
21 days	5.1 ± 3	96 ± 6**	56 ± 41
Pregnant + single iron supplement, 10 mg (n = 12)			
14 days	5.3 ± 0.4	421 ±43	43 ± 0.2
21 days	3.8 ± 1	192 ± 64**	66 ± 39
Control + single iron supplement, 10 mg (n = 12)			
14 days	5.1 ± 1.1	536 ± 72*	33 ± 2
21 days	4.3 ± 0.6	609 ± 25**	33 ± 14

Results are expressed as mean ± standard deviation

* $P \leq 0.05$, ** $P \leq 0.01$

0.51 ± 0.05 μg/mg protein respectively, by comparison to macrophages isolated from controls, 0.17 ± 0.07 μg/mg protein. Rats which received 125 mg iron during 4 weeks, or an iron deficient diet, had values for their alveolar macrophages of 0.39 ± 0.05 μg/mg protein and 0.09 ± 0.01 μg/mg protein, respectively.

Gene expression in macrophages

RT-PCR was utilized for the mRNA expression of each of the genes in the macrophage preparations. The expression of *DcytB* mRNA was not detectable in any of the macrophage preparations. *DMT1* was present only in iron deficient and control macrophages. *Ferroportin* mRNA expression was significantly altered by iron status, upregulated in iron deficiency and down regulated after iron loading, Fig. 1a. It was also significantly increased in macrophages isolated from both groups of pregnant rats. Macrophage *TfR-1* mRNA increased only in the iron deficient rats, all other animal groups showing no significant alteration in its expression, Fig. 1a.

Gene expression in enterocytes

Expression of these genes in enterocytes was also evaluated in the different animal groups. Using RT-PCR analysis, significant increases and decreases, respectively, in expression of *DcytB*, *DMT1*, *ferroportin* and *TfR-1* mRNA were identified in enterocytes isolated from rats either depleted or overloaded with iron (Fig. 1b). The imposition of pregnancy significantly increased DMT1 mRNA expression when analysed by either RT-PCR or real time PCR. Although, the mRNA expressions of both *DcytB* and *ferroportin* increased in the enterocytes isolated from the unsupplemented pregnant rats, when analysed by either RT-PCR or real tine PCR, these were not significant. *Transferrin receptor 1* (*TfR-1*) mRNA expression increased significantly in both pregnant groups, Fig. 1b.

Hepatic hepcidin levels

Figure 1c shows the levels of expression of *Hamp* in the livers of the two pregnant groups of rats as well as that of control rats supplemented or not with a single dose of iron analysed by real time PCR. Both pregnancy groups showed significant decreases in *Hamp* mRNA levels. Although the levels of hepatic iron increased 2-fold in the control animals administered one dose of iron, 10 mg, similar mRNA *Hamp* levels to that of controls were assayed.

Macrophage IRP activity

Figure 2 shows a typical band shift for alveolar macrophages isolated from controls, iron loaded and iron deficient rats when either 20 μg (1×) or 40 μg (2×) of cytosolic protein was analysed. Administration of β-mercaptoethanol, to convert IRP totally to the active form, did not greatly enhance the band intensity in the macrophages. No significant changes in IRP activity were evident in the macrophages isolated from either groups of pregnant rats. In contrast, the liver samples showed an enhancement in IRP activity after incubation with β-mercaptoethanol, as previously reported (Ward et al. 1994).

Macrophage immune function

Immune function was assessed in the different groups of macrophages by their ability to respond to different inflammatory stimuli, which activated NADPH oxidase, NFκB or inducible nitric oxide synthase, iNOS. Oxidative stress was evaluated by the assay of superoxide dismutase activity.

NADPH oxidase

Before stimulation of the macrophages, chemiluminescence was detectable in all macrophages, which reflected mitochondrial activity (Fig. 3). This was significantly elevated by low and high iron supplementation, 10 or 125 mg, as well as by pregnancy ± iron supplementation. After stimulation with either PMA or *fmlp* (not shown) there were significant increases in chemiluminescence in the macrophages from both groups of pregnant rats as well as the iron loaded groups. Macrophages isolated from iron deficient rats showed significantly decreased chemiluminescence both before and after stimulation in comparison to controls. This signal was inhibited by more than 95% in the presence of superoxide dismutase (not shown).

NFκB activation

Iron loading increased the latent activity of NFκB in the macrophages of both the pregnant and control rats (Table 4). Macrophages isolated from non-supplemented pregnant rats as well as iron deficient rats showed decreased values for latent NFκB activation compared to those of the controls. After in vitro activation with LPS + TNFα, there was enhanced macrophage NFκB activity in both the controls and pregnant rats, which had received iron. In contrast, macrophages isolated from non-supplemented pregnant rats and iron deficient rats showed a reduced ability to activate NFκB (Table 4).

Inducible nitric oxide synthase

Macrophage iNOS activity, as reflected by the formation of nitrite in the culture media, was enhanced or decreased in the non-supplemented or supplemented pregnant rats, respectively, by comparison to controls (Fig. 4) after in vitro stimulation of the macrophages with either LPS alone or LPS +IFNγ. Addition of either L-NAME or cyclohexamide to the incubation mixture, an NOS inhibitor and protein synthesis inhibitor respectively, during the stimulation time period, decreased NO release.

Superoxide dismutase activity

The mean activity of superoxide dismutase was enhanced significantly by both iron loading and pregnancy ± iron supplementation, which possibly indicated increased oxidative stress, Fig. 5.

Discussion

The increasing use of iron supplements by healthy individuals, as well as in pregnancy, in the belief that this will enhance the health of the mother, as well as that of the developing foetus, urgently requires investigation to ascertain that there are no adverse effects on important biochemical pathways. Furthermore, increased iron stores may increase the risk of

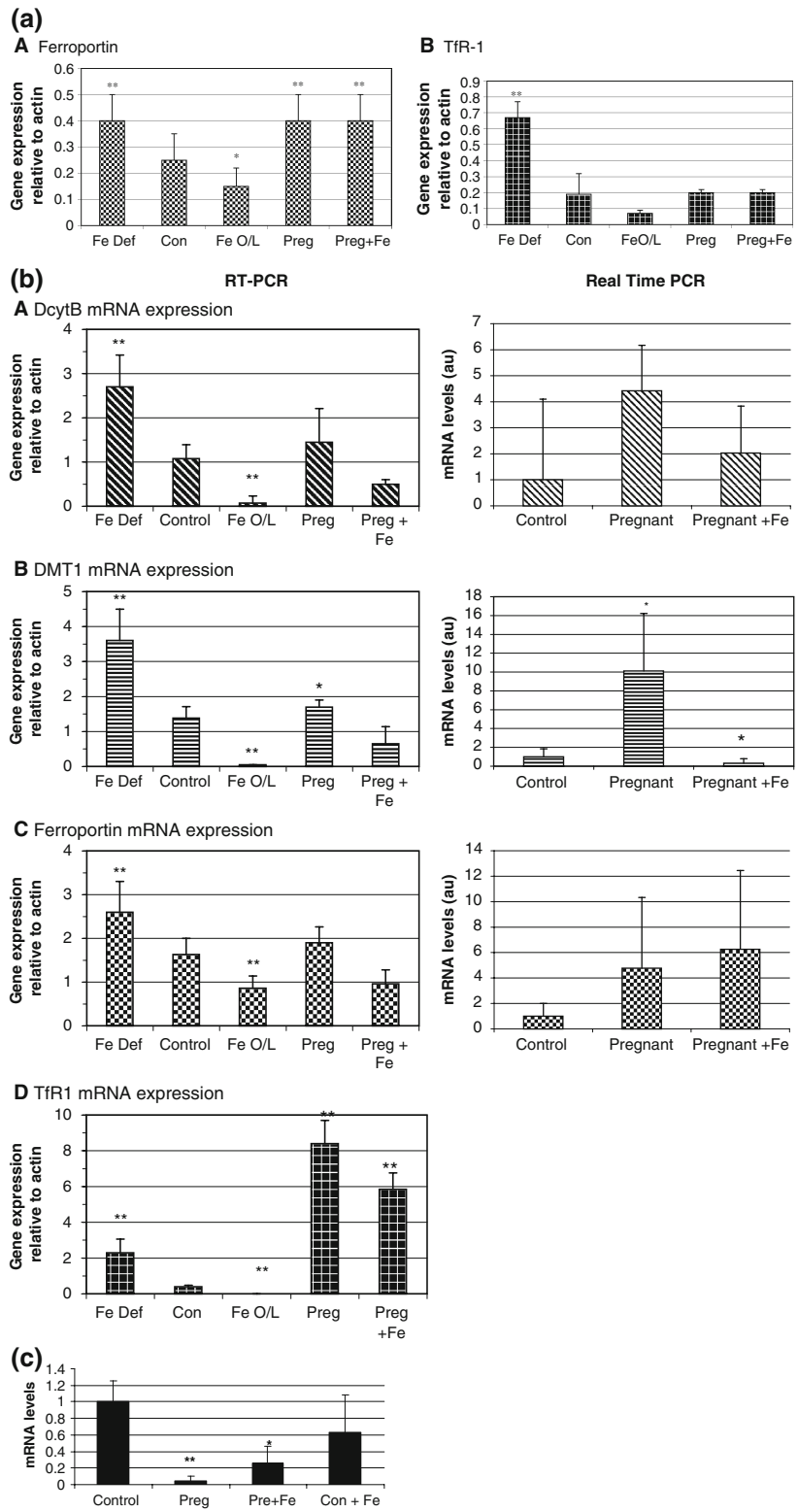

Fig. 1 (**a**) Macrophage *ferroportin* (A) and *TfR1*, (B) mRNA expression. Expression of each gene was measured by RT-PCR and the graph shows the mean results for all assays. Macrophages were isolated from 12 rats for each treatment group. Macrophages from three rats were pooled, approximately 3×10^6 cells, from which RNA was isolated such that each treatment group has four analyses. Data are mean ± standard deviation. Significant differences from controls * $P \leq 0.05$, ** $P \leq 0.01$. (**b**) Duodenal expression of *DcytB* (A), *DMT1* (B), *Ferroportin* (C) and *TfR1* (D). Expression of each gene was measured by RT-PCR, left hand side, and real time PCR right hand side. The graph shows the overall mean results for all assays. Results represent a minimum of six rats in each treatment group. Data are mean ± standard deviation for both RT-PCR and for real time PCR. Significant differences from controls * $P \leq 0.05$, ** $P \leq 0.01$. (**c**) Hepatic expression of *Hamp*. Expression of each gene was measured by real time PCR and the graph shows the overall mean results for all assays. A minimum of six rats is represented for each treatment group. Data are mean ± standard deviation. Significant differences from controls * $P \leq 0.05$, ** $P \leq 0.01$

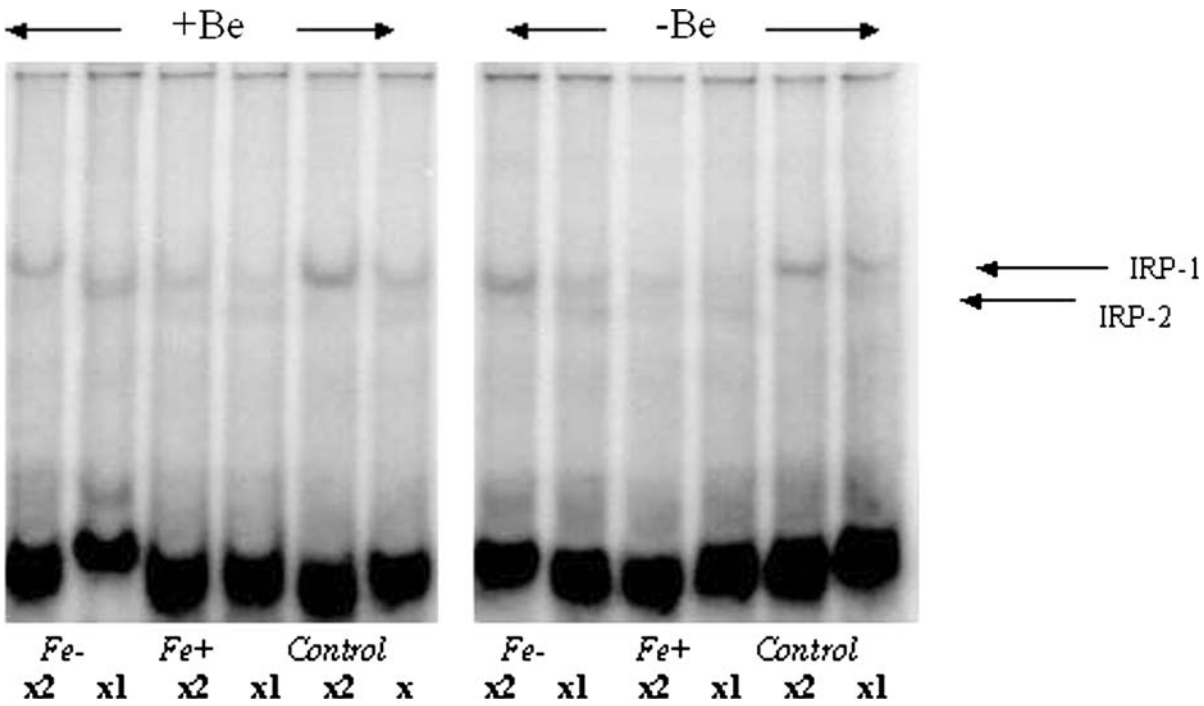

Fig. 2 IRP binding activity in chronically iron loaded, (+Fe), iron depleted, (−Fe) and control macrophages before, $-\beta$e, and after $+\beta$e, addition of 2% β-mercaptoethanol. 1× = 20 μg cytosolic protein, 2× = 40 μg cytosolic protein. Each analyses represent macrophages pooled from three rats

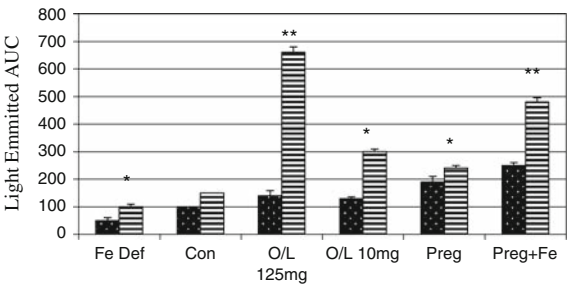

Fig. 3 Chemiluminescence in alveolar macrophages before (shaded dots) and after (shaded stripes) the addition of the stimulant. The Y axis represents area under the curve in arbitrary units. The results for iron deficient (Fe Def), controls (Con), chronic iron loading for 4 weeks (O/L 125 mg), a single 10 mg iron dose (O/L 10 mg) and in pregnant rats supplemented with a single 10 mg dose (Preg + Fe) or not (Preg) are presented. $n = 6$ in each group. The results are represented as mean ± standard deviation. Significant differences from controls * $P \leq 0.05$, ** $P \leq 0.01$

heart disease, cancer (such as breast cancer), and Alzheimer's disease.

One of the major objectives of this study was to study macrophage function in pregnant rats, which had an increased iron status. To this end, the animal

Table 4 NFκB activity in alveolar macrophages of rats of varying iron status before and after stimulation, in vitro, with LPS + TNFα for 30 min

	Macrophages (n = 12)	
	Unstimulated NFκB activity (% of controls)	NFκB activity after stimulation (% value in absence of stimulation)
Controls	100	134 ± 6
Iron loaded + 125 mg iron over 4 week period	279 ± 205**	124 ± 21
Iron loaded + single iron supplement, 10 mg	144 ± 10*	180 ± 30
Iron deficient	22 ± 4**	91 ± 4**
Pregnant	75 ± 76	94 ± 29*
Pregnant + single iron supplement, 10 mg	102 ± 5	159 ± 10

Results are presented as mean ± standard deviation

* $P \leq 0.05$; ** $P \leq 0.01$

For the NFκB experiments macrophages were pooled from three rats to obtain sufficient nuclear protein for parallel analyses of latent and activated NFκB activity. The analyses therefore represents four individual measurements

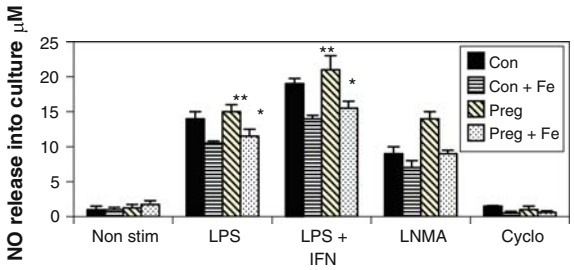

Fig. 4 Release of NO into the culture media after stimulation of alveolar macrophages from controls (Con), controls + a single dose of iron 10 mg after 21 days (Con + Fe), and pregnant rats of 21 days gestation, supplemented with iron on day 1 of pregnancy (Preg + Fe) or not (Preg). Stimulants used were lipopolysaccharide, LPS, and interferon gamma, IFN. N^G-nitro-L arginine, LNMA, and cyclohexamide, Cyclo, were used as inhibitors of iNOS and protein synthesis, and incubated with the cells during the stimulation period, respectively. Four rats in each treatment group. The experiment was carried out on three individual occasions and the figure shows a typical example of NO release in the different treatment groups. Results are presented as mean ± standard deviation. Significant differences from controls * $P \leq 0.05$, ** $P \leq 0.01$

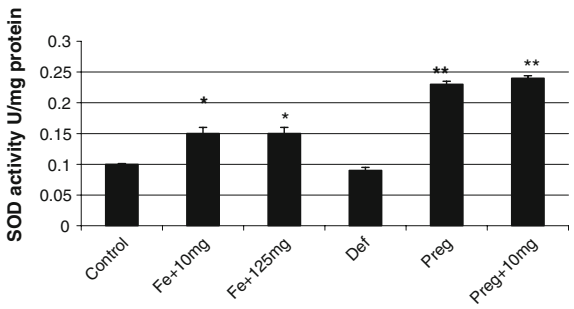

Fig. 5 Mean activities of superoxide dismutase in latent alveolar macrophages in controls, controls + single dose of iron 10 mg after 21 days, (Fe + 10 mg), chronically loaded rats + 125 mg iron dextran (Fe + 125 mg), and pregnant rats of 21 days gestation, supplemented with iron on day 1 of pregnancy (Preg + 10 mg) or not (Preg). Results are presented as mean ± standard deviation. Significant differences from controls * $P \leq 0.05$, ** $P \leq 0.01$

model used, was injected on day 1 with intramuscular iron, such that there was an increased iron status throughout the period of gestation, 21 days. Although a dietary preparation, such as carbonyl iron might be more comparable to the supplements used in human pregnancy, the major disadvantage would be that to achieve a significant elevation, of approximately 2-fold, over such a short time period, 21 days, would not be possible because of the mucosal block. Alveolar macrophages were used in these present studies of iron metabolism in pregnancy since they can be isolated as quiescent cells. Other reticulo-endothelial cells which could be isolated from this rat model, would be either peritoneal macrophages or Kupffer cells. However the former would be activated by the extraction process, such that the analysis of NOS and NADPH would not be possible, while the use of collagenase in the isolation of the latter would destroy RNA.

The growing foetus has a high demand for iron, which initially, will be derived from maternal sources. Once these are decreased, there must be a compensatory increase, both in maternal iron absorption via the enterocyte and also in iron transfer from the macrophages. Evidence for an increased maternal–foetal transfer and mobilisation of maternal iron in this present study was exemplified by the fall in maternal hepatic iron, decreases in certain haematological parameters, and significant changes in expression of specific genes associated with iron influx and efflux from enterocytes and macrophages, respectively.

The regulation of systemic iron balance is controlled by hepcidin, an anti-microbial peptide hormone, which is present in the circulation and produced, essentially, in the liver (Park et al. 2001). Its levels are altered mainly in response to changes in serum iron levels (Nemeth and Ganz 2006) although other factors such as hepatic iron content may have some influence. In this present study, the significantly reduced levels of haemoglobin and maternal iron stores in both pregnant groups, the latter only partially restored by a single iron supplement, resulted in significantly reduced hepatic *Hamp* mRNA expression, which if reflected at the protein level, would act as a signal of iron deficiency. It has been shown that ferroportin is the receptor for hepcidin (De Domenico et al. 2007), such that when hepcidin levels are reduced, ferroportin activity will enhance the efflux of iron from the macrophages. Indeed, in this present study, *ferroportin* mRNA expression increased in both pregnancy groups. In contrast, when iron levels are raised, ferroportin will bind to hepcidin, be internalised and degraded, thereby reducing iron efflux from the macrophage (Nemeth et al. 2004).

Enterocytes and macrophages can take up and release iron; enterocytes can absorb both dietary haem and non haem iron, while macrophages are involved in the acquisition of effete red blood cells, via phagocytosis, as well as processing of iron for erythropoiesis. It was noted that the iron content of

the macrophages isolated from the supplemented and non-supplemented rats were high in comparison to those macrophages isolated from iron loaded rats. The explanation for this is unclear but could be related to the high expression of TfR1 mRNA expression identified. Iron deficiency and iron overload induced significant alterations in the expression of *DcytB*, *DMT*, *ferroportin* and *TfR-1* in enterocytes (present study). This has been previously reported by Dupic et al. (2002) in mice and in IEC-6 cells (Thomas and Oates 2002). *Ferroportin* and *TfR-1* mRNA levels also increased in the macrophages studied. It was of note that *DMT1* mRNA increased in the duodenal enterocytes of pregnant rats, while the expression of the other genes analysed by either RT-PCR or real time PCR showed no significant changes. A similar lack of changes in these genes in non-supplemented pregnant rats was also reported by Millard et al. (2004) although in this study *DcytB* mRNA expression was significantly increased by pregnancy. In an earlier study, by Batey and Gallagher (1977), increased iron absorption was identified in iron overloaded pregnant rats, although not to the same extent as non-supplemented pregnant rats. Such results indicate that iron dependent and iron independent factors, e.g. oestrogens, are involved in iron absorption during pregnancy.

Macrophages isolated from rats, which had been chronically iron loaded or depleted in vivo, did not show a significant inverse relationship with IRP activity. This is in contrast to immortalised macrophage cell lines such as RAW 264.7, where incubation with desferrioxamine caused a significant increase in IRP-1 binding activity, as well as slightly increasing IRP-2 activity (Kim and Ponka 2000) To what extent the IRP/IRE system may play a role in post translational regulation of certain mRNAs in macrophages remains unclear, since there were no changes in binding activity before or after exposure to 2% β-mercaptoethanol. This could reflect the fact that animal derived alveolar macrophages are non-proliferating cells, and have low levels of transferrin receptors. In contrast, immortalised cell lines have consistently higher levels of transferrin receptors compared to the corresponding animal derived cells. This reflects the fact that their principal pathway of iron uptake is from transferrin, whereas the main route for iron uptake into macrophages is by phagocytosis of effete red blood cells.

During pregnancy, a reduction in the inflammatory response has been reported, caused by a variety of factors including high oestrogen levels (Carr 1990), reductions in the stimulated release of arachidonic acid (AA) and leukotriene B_4 (LTB_4), and of NADPH oxidase activity (Crocker et al. 2001) in order to maintain a physiologically compatible environment for the foetus (Raghupathy 1997; Dealtry et al. 2000; Mellor and Munn 2000). Latent macrophage NADPH oxidase activity increased in both pregnant groups, regardless of iron supplementation in these present studies, which is in contrast with human pregnancy where the activity of this enzyme is diminished (Crocker et al. 2001). This could relate to the high concentration of iron determined in these cells. Further studies are needed to investigate the speciation of the iron present. Only during pre-eclampsia is an increased activity of this enzyme reported in human pregnancy (Dechend et al. 2003). In some studies it has been suggested that this enzyme may be involved in the activation of NFκB in certain cell types (Bowie and O'Neill 2000). However, no relationship with NFκB activity was apparent, with respects to either its latent or stimulated activity in these present studies.

It was noteworthy that NFκB activity was reduced in macrophages isolated from pregnant rats which had not received the iron supplement and that there was little further activation after stimulation. In contrast, a single iron supplement restored the activation of NFκB to values comparable to control. Nuclear factor-κB is a family of ubiquitously expressed transcription factors that are recognised as critical regulators of the mammalian immune and inflammatory responses. The NFκB complex is found in almost all cells and it is generally recognized as an essential cell mediator. Activation of NFκB occurs in response to extracellular chemical stresses, various cytokines and growth stimuli, which results in the direct induction of hundreds of genes, whose cellular influences extend well beyond those of the immune system.

Macrophages isolated from pregnant rats showed increased NO release after in vitro stimuli, despite their lower activation of NFκB. In contrast when the iron supplement was administered, iNOS was decreased. In macrophages there is an autoregulatory feedback loop that links iron homeostasis with optimal formation of NO for host defence. Both in vivo and in vitro iron loaded cells, (Zhang et al. 1998; Dlaska and Weiss 1999), respectively, show a reduction in NO release

after challenge with LPS and INF-γ. However, the underlying mechanism by which iron exerts transcriptional regulation of iNOS has remained elusive although recent data suggest that activation of hypoxia inducible factor 1 (HIF-1) may be involved (Lu et al. 2006).

Pregnancy is a condition that favours oxidative stress; serum lipid peroxidation products are increased in normal pregnant women, particularly during the second trimester (Qanungo and Mukherjea 2000; Uotila et al. 1991) while other cytoprotective enzymes and antioxidants are also elevated (Casanueva and Viteri 2003). Enhanced activities of superoxide dismutase were assayed in latent alveolar macrophages isolated from pregnant rats ± iron supplement on day 21. Erythrocyte superoxide dismutase activity is decreased by pregnancy, (Wisdom et al. 1991; Ibouno et al. 1996) due to changes in red cell physiology associated with haemodilution (Mukhopadhyay et al. 2004). The increases in the iron content of the macrophages isolated from the two pregnant groups may have contributed to the higher SOD activity.

In human studies involving non anaemic pregnant women from Mexico City, iron supplements, 60 mg/day, increased the risk of haemoconcentration, low birth weight and premature birth (Casanueva and Viteri 2003). In another study, daily supplements of 100 mg Fe + vitamin C were administered to 27 healthy women in the third trimester of pregnancy and compared with 27 healthy non-supplemented controls. Delivery occurred at an earlier stage in the supplemented group, 37 weeks compared to 39 weeks, while serum iron was significantly higher and vitamin E status was lower (Lachili et al. 2001). Such studies must question whether the use of iron supplements in pregnancy is compatible with a successful pregnancy.

There is an increasing use of different supplements by the general population in the belief that beneficial results will be attained. However this study has revealed that marginal increases in iron status, which could be readily achieved in normal subjects, who had increased their iron intake, (either from iron fortified food or additional iron supplements), may have detrimental effects, particularly on the maternal immune responses. Further studies need to be undertaken to identify the benefits and adverse effects of iron supplementation, however small, during pregnancy.

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References

Batey RG, Gallagher ND (1977) Role of the placenta in intestinal absorption of iron in pregnant rats. Gastroenterol 72:255–257

Beauchamp C, Fridovich I (1971) Superoxide dismutase improved assays and an assay applicable to acrylamide gels. Anal Biochem 44:276–287. doi:10.1016/0003-2697(71)90370-8

Bowie LA, O'Neill LA (2000) Oxidative stress and nuclear factor-kappaB activation: a reassessment of the evidence in the light of recent discoveries. Biochem Pharmacol 59:13–23. doi:10.1016/S0006-2952(99)00296-8

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal Biochem 72:248–254. doi:10.1016/0003-2697(76)90527-3

Carr RB (1990) The fetal maternal placental unit. In: Becker KL (ed) Principles and practice of endocrinology and metabolism. J.B. Lippincott, pp 788

Casanueva E, Viteri FE (2003) Iron and oxidative stress in pregnancy. Am Soc Nutr Sci 133:1700S–1708S

Crichton RR, Wilmet S, Legssyer R, Ward RJ (2002) Molecular and cellular mechanisms of iron homeostasis and toxicity in mammalian cells. J Inorg Biochem 91:9–18. doi:10.1016/S0162-0134(02)00461-0

Crocker IP, Lawson PN, Baker PN, Fletcher J (2001) The anti-inflammatory effects of circulating fatty acids in obstructive jaundice: similarities with pregnancy-induced immunosuppression. QJM 94:475–484. doi:10.1093/qjmed/94.9.475

Dealtry GB, O'Farrell MK, Fernandez N (2000) The Th2 cytokine environment of the placenta. Int Arch Allergy Immunol 123(2):107–119. doi:10.1159/000024441

Dechend R, Viedt C, Muller DN, Ugele B, Brandes RP, Wallukat G et al (2003) AT1 receptor agonistic antibodies from pre-eclamptic patients stimulate NADPH oxidase. Circulation 107:1632–1639. doi:10.1161/01.CIR.0000058200.90059.B1

De Domenico I, Ward DM, Langelier C, Vaughn MB, Nemeth E, Sundquist WI et al (2007) The molecular mechanism of hepcidin-mediated ferroportin down-regulation. Mol Biol Cell 18:2569–2578. doi:10.1091/mbc.E07-01-0060

Dlaska M, Weiss G (1999) Central control of transcription factor NF-IL6 for cytokine and iron mediated regulation of murine inducible nitric oxide synthase expression. J Immunol 162:6171–6177

Dupic F, Fruchon S, Bensaid M, Loreal O, Brissot P, Borot N et al (2002) Duodenal mRNA expression of iron related genes in response to iron loading and iron deficiency in four strains of mice. Gut 51:648–653. doi:10.1136/gut.51.5.648

Ibouno LE, Shu EN, Igbokwe GE (1996) An improved technique for the assay of red blood cell superoxide dismutase (SOD) activity. Clin Chim Acta 247:1–6. doi:10.1016/0009-8981(95)06193-2

Kim S, Ponka P (2000) Effects of interferon-γ and lipopolysaccharide on macrophage iron metabolism are mediated by nitric oxide-induced degradation of iron regulatory protein 2. J Biol Chem 275:6220–6226. doi:10.1074/jbc.275.9.6220

Kustermans G, El Benna J, Piette J, Legrand-Poels S (2005) Perturbation of actin dynamics induces NF-kappaB activation in myelomonocytic cells through an NADPH oxidase-dependent pathway. Biochem J 387:531–540. doi:10.1042/BJ20041318

Lachili B, Hininger I, Faure H, Arnaud J, Richard MJ, Favier A et al (2001) Increased lipid peroxidation in pregnant women after iron and vitamin C supplementation. Biol Trace Elem Res 83:103–110. doi:10.1385/BTER:83:2:103

Legssyer R, Ward RJ, Crichton RR (1999) Effect of chronic chloroquine administration on iron loading in the liver and reticuloendothelial system and on oxidative responses by the macrophage. Biochem Pharmacol 57:907–911. doi:10.1016/S0006-2952(98)00368-2

Legssyer R, Josse C, Piette J, Ward RJ, Crichton RR (2003) Changes in function of iron-loaded alveolar macrophages after in vivo administration of desferrioxamine and/or chloroquine. J Inorg Biochem 94:36–42. doi:10.1016/S0162-0134(02)00633-5

Lu DY, Liou HC, Tang CH, Fu WM (2006) Hypoxia-induced iNOS expression in microglia is regulated by the PI3-kinase/Akt/mTOR signaling pathway and activation of hypoxia inducible factor-1alpha. Biochem Pharmacol 72:992–1000. doi:10.1016/j.bcp. 2006.06.038

Mellor A, Munn DH (2000) Immunology at the maternal-fetal interface: lessons for T cell tolerance and suppression. Annu Rev Immunol 18:367–391. doi:10.1146/annurev.immunol.18.1.367

Millard K, Frazer D, Wilkins SJ, Anderson GR (2004) Changes in the expression of intestinal iron transport and hepatic regulatory molecules explain the enhanced iron absorption associated with pregnancy in the rat. Gut 53:655–660. doi:10.1136/gut.2003.031153

Mukhopadhyay A, Bhatla N, Kriplani A, Agarway N, Saxena R (2004) Erythrocyte indices in pregnancy: effect of intermittent iron supplementation. Natl Med J India 17:135–137

Myatt L, Cui X (2004) Oxidative stress in the placenta. Histochem Cell Biol 122:369–382. doi:10.1007/s00418-004-0677-x

Nemeth E, Ganz T (2006) Regulation of iron metabolism by hepcidin. Annu Rev Nutr 26:323–342. doi:10.1146/annurev.nutr.26.061505.111303

Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM et al (2004) Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 306:2090–2093. doi:10.1126/science.1104742

Oppenheimer SJ (2001) Iron and its relation to immunity and infectious disease. J Nutr 131:616S–635S

Park CH, Valore EV, Waring AJ, Ganz T (2001) Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem 276:7806–7810. doi:10.1074/jbc.M008922200

Qanungo S, Mukherjea M (2000) Ontogenic profile of some antioxidants and lipid peroxidation in human placental and fetal tissues. Mol Cell Biochem 215:11–19. doi:10.1023/A:1026511420505

Raghupathy R (1997) Th1-type immunity is incompatible with successful pregnancy. Immunol Today 18(10):478–482. doi:10.1016/S0167-5699(97)01127-4

Rioux FM, LeBlanc CP (2007) Iron supplementation during pregnancy: what are the risks and benefits of current practices? Appl Physiol Nutr Metab 32:282–288. doi:10.1139/H07-012

Stolzfus R, Dreyfuss M (1988) Guidelines for the use of iron supplements to prevent and treat iron deficiency anaemia. International nutritional anaemia consultative group. ILSI Press, Washington

Thomas C, Oates PS (2002) IEC-6 cells are an appropriate model of intestinal iron absorption in rats. J Nutr 132:680–687

Uotila J, Tuimala R, Aarnio T, Pyykko K, Ahotupa M (1991) Lipid peroxidation products, selenium-dependent glutathione peroxidase and vitamin E in normal pregnancy *Eur J Obstet*. Reprod Biol 42:95–100

Ward RJ, Kuhn L, Kaldy P, Florence A, Peters TJ, Crichton RR (1994) Control of cellular iron homeostasis by Iron Responsive Element (IRE) in vivo. Eur J Biochem 220:927–931. doi:10.1111/j.1432-1033.1994.tb18696.x

Ward RJ, Zhang Y, Crichton RR, Biret B, Piette J, De Witte P (1996) Expression of NFKappaB in rat brain after administration of ethanol in vivo. FEBS Lett 389:119–122. doi:10.1016/0014-5793(96)00545-5

Ward RJ, Wilmet S, Legssyer R, Crichton RR (2004) Iron supplementation during pregnancy-a necessary or toxic supplement. Bioinorg Chem Appl 1:169–176. doi:10.1155/S156536330300013X

Wisdom S, Wilson R, McKillop JH, Walker JJ (1991) Antioxidant systems in normal pregnancy and in pregnancy-induced hypertension. Am J Obstet Gynecol 165:170–174

WHO (2001) WHO/NHD/01.3. Geneva

Zhang Y, Crichton RR, Boelaert JR, Jorens PG, Herman AG, Ward RJ et al (1998) Decreased release of nitric oxide by alveolar macrophages after in vivo loading with iron or ethanol. Biochem Pharmacol 55:21–25. doi:10.1016/S0006-2952(97)00382-1