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The Effect of Iron Deficiency on Osmotic Sensitivity of Red Blood Cells from Neonatal Rats and Their Mothers

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Abstract Iron deficiency during pregnancy has many effects on both the mother and her developing foetus. These can be both short and long term. One effect is an alteration in fatty acid metabolism and we hypothesised that these changes may result in alterations in membrane function and structure. In order to test this hypothesis, we measured osmotic sensitivity in red blood cells isolated from neonates and their mothers at different times following birth. We fed female rats control or iron-deficient diets for 4 weeks prior to mating and kept them on the same diet until term. At that time, we returned one group of deficient dams to the control diet. The others were kept on the same diet. We showed that iron deficiency results in a decrease in osmotic sensitivity in the mothers but not in their neonates. Returning the dams to the control diet resulted in a return of their red cell osmotic sensitivity to control levels. In the neonates, there was no recovery in haematocrit or in any other parameter, though they did not get any worse, in contrast to the pups being suckled by deficient mothers. The data show two things. The first is that following birth, the mother restores her own iron stores at the expense of the pups, and secondly, there are differences in properties and sensitivities between red cells from mothers and their neonates. This latter observation cannot be explained by differences in the membrane fatty acid profiles, which were not significantly different.

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Abbreviations

BSS	Balanced salt solution
FAME	Fatty acid methyl esters
Hct	Haematocrit
Hb	Haemoglobin
Hb/cell	Haemoglobin concentration per cell
LC_{50}	50 % lysis concentration
MCV	Mean cell volume
OD	Optical density
RBC	Red blood cell

Introduction

Iron deficiency anaemia is a global public health problem affecting both developing and developed countries, with major consequences for human health. It is the most common nutritional deficiency worldwide (Killip et al. 2007). Iron deficiency anaemia occurs at all stages of the life cycle, but is most prevalent in pregnant women and young children. Iron deficiency during pregnancy and/or lactation has serious consequences for both the mother and her offspring. In humans and animals, studies have shown that iron deficiency during pregnancy can cause preterm delivery, small birth size and increased mortality in both mother and child. In animal models, the long-term consequences include hypertension, obesity and dyslipidemia (Lewis et al. 2002; Gambling et al. 2003). In humans, low iron status during pregnancy leads to an increased placental: fetal ratio (Barker 1992) which can be used as a

predictor of cardiovascular disease in later life (McArdle and Ashworth 1999). More recently, observational studies have suggested that low iron status, especially in early pregnancy, can result in reduced size at birth (Alwan et al. 2010, 2011), while intervention studies have shown that iron supplementation can increase birth weight, even in women of apparently normal iron status (Cogswell et al. 2003).

One finding consistent between our rat model and other models of iron deficiency during pregnancy is a change in lipid profile and signs of dyslipidemia (Lewis et al. 2001; Zhang et al. 2005). This led to the hypothesis that one consequence of iron deficiency could be a change in the fatty acid profile of cells, with a consequent alteration in cellular function. This could then explain why there is such a diverse offspring phenotype associated with iron deficiency during pregnancy, ranging from hypertension and obesity through to neurological changes and developmental delay.

In order to test this hypothesis, we have examined the effect of iron deficiency on osmotic fragility in red blood cells (RBCs). We have used osmotic sensitivity as a marker for membrane fragility (Orcutt et al. 1995) and have examined erythrocytes from both the dams and the neonates at different times after birth. RBCs were chosen for this study, as they are a cell with well-defined characteristics and a substantial literature. They are also easily obtainable and provide a simple and robust assay system (McMullin 1999; An and Mohandas 2008).

Materials and Methods

Experimental Diets

Experimental diets were based on a dried egg albumin. $FeSO_4$ was added to achieve levels of added iron of 5 mg kg^{-1} for control diet (C) and 7.5 mg kg^{-1} for iron-deficient diet (FD). Dietary ingredients were purchased from Fisher Chemicals (UK), Sigma (UK) and Igraca (France).

Experimental Animals

All experimental procedures were approved and conducted in accordance with the UK Animal (Scientific Procedures) Act, 1986. Experiments were performed using weanling female Rowett Hooded Lister rats. 94 animals were used. All animals were fed control diet (C) for 2 weeks, and then divided into two groups. The first group remained on the control diet throughout the experiment. The second group was placed on an iron-deficient diet (FD) for 4 weeks before mating. All rats were then mated with males of the same strain. Mating was confirmed by detection of a vaginal plug. At birth, eight pups (all males when possible) from each litter were kept with their mothers. The remaining pups were culled and blood samples were collected. At this stage, a subgroup of the mothers on the iron-deficient diet was changed to the control diet. The rest were kept on the same diet. At different times, ranging from 0 to 10 days after birth, eight mothers and their pups were killed, and the blood and tissue samples were collected. In order to determine whether effects seen in the dam were a consequence of pregnancy or of iron deficiency, a second, parallel experiment with non-pregnant females was carried out. These females were given the same diets, for the same length of time, and were killed at the same age as the 10 day post-pregnancy females. Blood and tissue samples were collected.

Blood Sample Collection

Maternal, non-pregnant female and litter whole blood was collected in EDTA-coated tubes. Blood was collected from all pups in a litter, and pooled, in order to obtain a representative neonatal blood sample, 100 µl of whole blood from each litter and mother was added to 1 ml of phosphate-buffered saline (PBS) with 5 mM glucose, and stored at 4 °C until analysed (24–48 h later). The remaining whole blood was then centrifuged; the plasma was collected and stored at −80 °C. The remaining RBCs were stored at 4 °C until analysed (up to 1 h).

Haematological Measurements

Maternal and neonatal haematocrits (Hct) were measured by drawing blood into capillary tubes, which were then centrifuged in a high-speed Hct centrifuge and read in a microhematocrit reader. Haemoglobin (Hb) concentration was measured using Quantichrom Hemoglobin assay kit (Bioassay Systems, USA). RBC analysis was carried out using a Sysmex KX21N cell counter.

Osmotic Fragility Measurement

The whole blood stored in PBS and glucose was washed twice and diluted at 1:10 with ice cold PBS. NaCl lysis buffer was prepared at different milliosmolar concentrations, ranging from 310 to 100 mOsm L^{-1}. 10 µl of the blood was incubated with 200 µl of each of the decreasing mosmolar concentration for 1–2 min. All the samples were analysed at room temperature (22–25 °C). The samples were then centrifuged at high speed, and the supernatants were collected. The supernatants were read at 540 nm, and the 50 % lysis concentration (LC_{50}) was then determined.

RBC Ghost Preparation

The stored RBCs were washed in ice cold balanced salt solution (BSS). Lysis buffer was prepared containing 25 mM NaCl, 10 mM Hepes, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. The pellet was resuspended in the lysis buffer (ice cold) and centrifuged at $4000\times g$ for 5 min at 4 °C. The supernatant was discarded. The pellets was washed in the lysis buffer a further twice or until the supernatant is clear.

Extraction of Lipid Fractions from RBC Ghosts

10 µl of RBC ghosts was resuspended in 0.24 ml of ice cold water and ultrasonicated. 10 µl of C19:00 fatty acid internal standard (2 mg ml^{-1}) was added to the sample, followed by 1.2 ml of methanol: chloroform containing butylated hydroxytoluene (2:1). The samples were then incubated in a bath sonicator for 30 min. Centrifuged at $8800\times g$ for 15 min, and the top, aqueous layer was discarded. The organic layer was collected into a clean vial. The remaining pellet was resuspended in 1 ml of chloroform: methanol: water (4:8:3). Centrifuged at $8800\times g$ for 15 min, the organic layer was collected into the same vial. This extraction step was repeated with 1 ml of the following chloroform: methanol (1:1) and chloroform: methanol (2:1). 50 µl of 0.88 % KCl was added to the collected organic layer and centrifuged at $1000\times g$. The top aqueous layer was aspirated, and 1 g of anhydrous sodium sulphate was added to the remaining organic layer. This mixture was then centrifuged at $1000\times g$, and then the organic layer was transferred to a clean vial and dried under nitrogen at 40 °C.

Fatty Acid Methyl Esters (FAME) Analysis

Fatty acids were measured using derivatisation followed by gas chromatography. Samples were dissolved in hexane and methanolic hydrochloric acid, together with C17:0 internal standard. The samples were flushed with nitrogen, capped and heated at 100 °C for 2 h. After cooling, dH_2O and hexane were added. The samples were mixed and centrifuged ($1000\times g$, 1 min) to separate the layers. The top layer was removed and the remainder was re-extracted. This procedure was repeated twice. Water was then added to the hexane, mixed, centrifuged and aspirated. This procedure was repeated. Anhydrous sodium sulphate was added and mixed. The organic layer was dried under nitrogen, and the residue was dissolved into 0.02 % BHT in hexane. The samples were then analysed by gas chromatography and the data were normalised to the 17:00 internal standard. The results are the mean ± M of at least six measurements.

Atomic Absorption Spectrophotometric Analyses

For the estimation of liver tissue Fe, samples were dried at 100 °C, and then wet-ashed with nitric acid (Ultrapure; Merck, Poole, U.K.). The Fe content was determined by graphite furnace atomic spectrophotometry (AAnalyst 600; Perkin-Elmer, USA). A standard curve for Fe was prepared from commercially available standards (Spectrosol; BDH, Poole, UK). Appropriate quality controls were included as necessary.

Statistical Analysis

For each mother, the litters were averaged and data were recorded as a single point. This is instead of treating each offspring as a single point, as this is statistically more accurate option. All results are presented as mean ± SEM. Data were analysed by two-way ANOVA with dietary treatments being compared with a post hoc t test using GraphPad Prism 5. Significance was assumed at $p \leq 0.05$.

Results

Weights and Survival

There was no effect of iron deficiency on maternal weights at either time points measured. At birth, neonates from iron-deficient mothers were smaller than those from control mothers. By day 10, they were still significantly smaller (Fig. 1). 10 % of pups born to iron-deficient mothers died at birth. None of the pups were born to control mothers died.

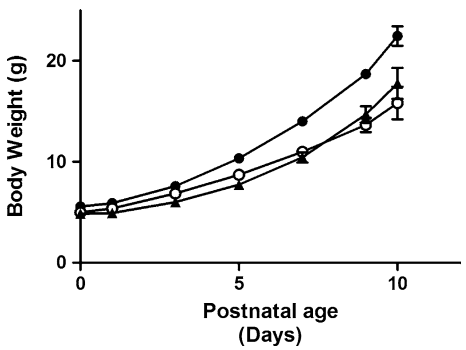

Fig. 1 The effect of maternal iron deficiency on growth in the neonates. Dams were given either control (*closed circles*), iron deficient (*open circles*) or iron deficient followed by control at birth (*closed triangles*) diets. Pups were weighed at each of the specified days and the mean value was taken for each litter. The data are the mean ± SEM of at least six litters

Haematological and Iron Measurements

As would be expected, liver iron levels in the mother were lower at birth in animals given iron-deficient diets than controls (Fig. 2a). The iron levels in mothers on control diet remained higher than animals kept on a deficient diet, and also, interestingly, there was an increase in levels in those returned to a control diet following giving birth. In the offspring, iron stores were higher in the controls. This did not change (Fig. 2b) in either the offspring whose mothers were returned to control diets or those kept on deficient diets. In all three groups, iron levels dropped as postnatal development proceeded.

A different pattern was seen for the Hct (Fig. 3). In the mothers, those animals returned to a control diet recovered their Hct rapidly, returning to control values by day 7 (Fig. 3a). Returning the mothers to a control diet allowed the offspring to maintain, but not improve, their Hct (Fig. 3b).

Similar results were seen in the mean cellular volume (MCV) and in the haemoglobin (Hb) content per cell (Fig. 4). Returning mothers to a normal diet allowed a return to the same values as the controls (Fig. 4a, c), while the neonates showed no improvement, though those suckling from mothers returned to a control diet did not get any worse compared to their control counter parts (Fig. 4b, d).

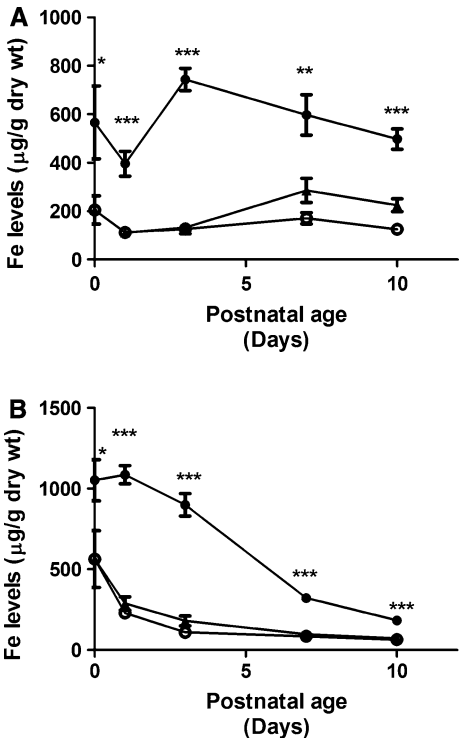

Fig. 2 The effect of maternal iron deficiency on iron levels in the liver of maternal rats (**a**) or their pups (**b**) taken at different stages after birth. Dams were given either control (*closed circles*), iron deficient (*open circles*) or iron deficient followed by control at birth (*closed triangles*) diets. Groups of eight animals were killed at each stage. The data are the mean ± SEM of at least six animals at each stage in development. ***$p < 0.001$

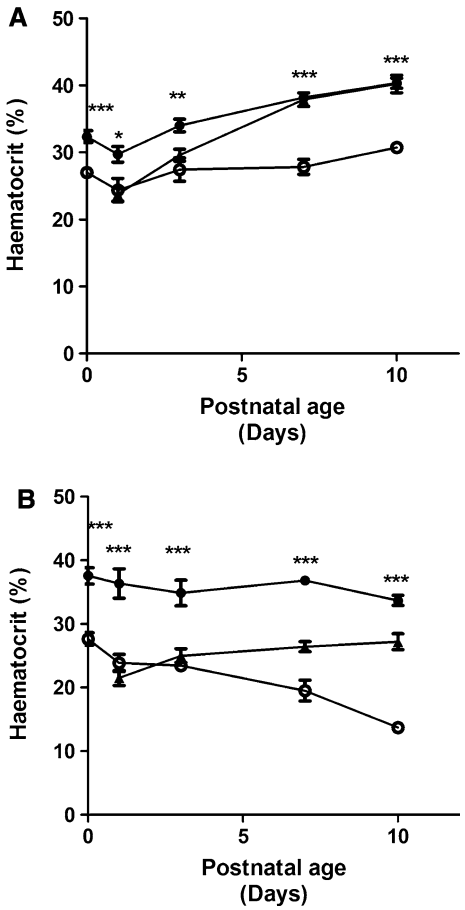

Fig. 3 The effect of maternal iron deficiency on haematocrit of maternal rats (**a**) or their pups (**b**) taken at different stages after birth. Dams were given control (*closed circles*), iron deficient (*open circles*) or iron deficient followed by control at birth (*closed triangles*) diets. Groups of eight animals were killed at each stage. The data are the mean ± SEM of at least six animals at each stage in development. ***$p < 0.001$

Osmotic Fragility

The sensitivity of erythrocytes to low osmotic pressure was measured by determining the milliosmolar concentration required to produce 50 % cell lysis (LC_{50}). A representative example of the data obtained is given in Fig. 5. In the dams at day 0, the iron-deficient erythrocytes LC_{50} was lower compared to control erythrocytes ($p < 0.01$, Fig. 6a). This difference remained between controls and dams who continued to be given the iron-deficient diet or those returned to control (Fig. 6a), consistent with a relatively slow turnover of red cells. In contrast, there was no

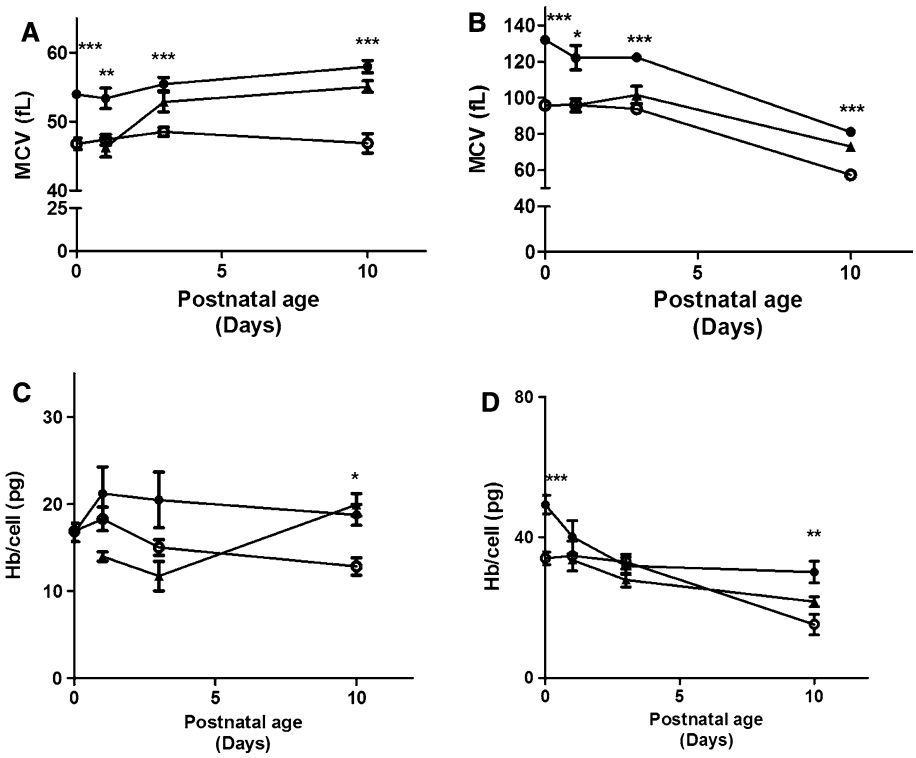

Fig. 4 The effect of maternal iron deficiency on haematological parameters of maternal rats (**a**, **c**) or their pups (**b**, **d**) taken at different stages after birth. Dams were given control (*closed circles*), iron deficient (*open circles*) or iron deficient followed by control at birth (*closed triangles*) diets. Groups of eight animals were killed at each stage. The data are the mean ± SEM of at least six animals at each stage in development. ***$p < 0.001$

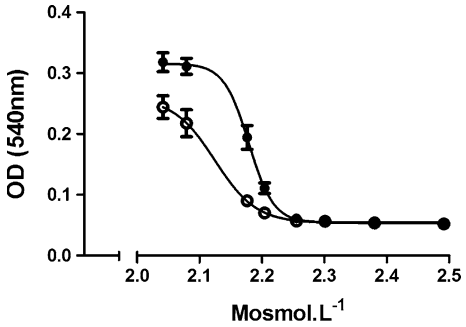

Fig. 5 The effect of maternal iron deficiency on osmotic sensitivity of red cells. This is an example of data used to calculate Fig. 6. The cells were incubated in solutions of decreasing osmotic pressure as described in "Materials and Methods" section. The release of Hb was measured at 540 nm. The results are the mean ± SEM of at least six animals and are plotted as $\log_{10}$ of the osmotic pressure

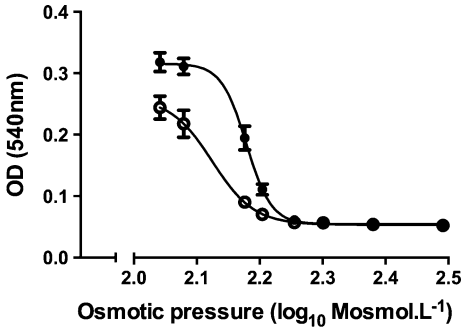

Fig. 6 The effect of maternal iron deficiency during pregnancy on osmotic sensitivity of red cells taken from mothers (**a**), their offspring (**b**), or female rats that were not pregnant but were given the same diets and killed at the same age as the 10 days post partum mothers $n = 3$ and 5; *$p < 0.05$ (**c**). The dams were fed a control (*closed circles*), iron deficient (**b**) or iron deficient followed by control at birth (*closed triangles*) diets. The animals were killed at the given days and the red cells isolated and treated as described in "Materials and Methods" section. The results are the mean ± SEM of at least six animals. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

significant difference between erythrocytes any of the groups of neonatal animals (Fig. 6b). To test whether the effect seen in the dams was related to pregnancy or iron deficiency specifically, we carried out osmotic sensitivity experiments in RBC from control and iron-deficient non-pregnant female rats, with the same outcome ($n = 3$ and 5; $p < 0.03$, Fig. 6c).

Fatty Acid Profile of RBC Membranes

Fatty acid profiles were measured in RBC ghosts from dams and neonates at day 0 and day 10 after birth. As described in "Materials and Methods" section, the results from any one run were normalised to the fatty acid standards (C17:00). Although there were slight trends in differences ($p = 0.08$), analysis of the levels of total saturated and unsaturated fatty acids did not achieve significance (Fig. 7).

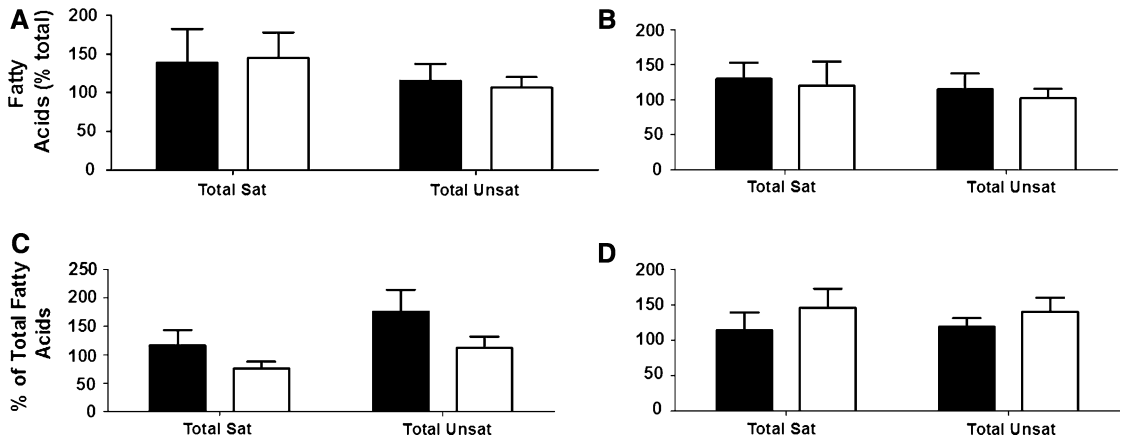

Fig. 7 The effect of maternal iron deficiency during pregnancy on red blood cell fatty acid profiles in mothers (**a**, **b**) and their offspring (**c**, **d**) taken at day 0 (**a**, **c**) and 10 days (**b**, **d**) post partum. Closed histograms are taken from controls, *open bars* from iron-deficient dams. The results are the mean ± SEM of at least six independent readings

Discussion

This study has examined the effect of maternal iron deficiency during pregnancy on iron status and osmotic fragility of the erythrocytes in both the mothers and their neonates. Our work extends previous studies, relating growth, development and gene expression in rats made iron deficient during pregnancy (Gambling et al. 2009). The results of this study show that there is a hierarchy of importance in utilisation of iron following birth. During pregnancy, iron is directed primarily to the developing foetus, with the maternal haematocrit being allowed to fall (Gambling et al. 2009). The opposite happens after birth. The mothers' haematocrit rises rapidly when she is returned to a normal diet, returning to control values within 3 days after birth. In contrast, maternal liver iron stores do not recover, even after 10 days.

Returning the mother to a normal iron diet stops any further decrease in the neonatal haematocrit, but there is no recovery over the 10-day period of this experiment. Iron stores in the neonate suckling from a mother returned to the control diet follow the same pattern as those still with a deficient dam, but they do manage to maintain their haematocrit levels during this suckling period. Thus, we can conclude from these results that the priority for iron supply has returned to the mother after birth. This change fits with the observation that iron levels in the maternal milk are dramatically reduced by maternal iron deficiency (Kelleher and Lonnerdal 2005).

Our data show that maternal iron deficiency does affect the function of maternal erythrocyte membranes but surprisingly, maternal iron deficiency does not affect the neonatal erythrocyte membranes in the same way. This sensitivity to iron status is not the product of pregnancy alone, since the same observation is made in non-pregnant animals. The data give rise to two questions: why are maternal RBC altered by iron deficiency and why are the neonatal red cells not affected in the same way?

Using a variety of different techniques, many authors have shown that iron deficiency is associated with changes in red blood cell structure and function. Several studies have shown small changes in the fatty acid make up and cholesterol content of the red blood cell membranes (Cunnane and McAdoo 1987). Our data do not support these observations, most likely because the degree of anaemia induced in our model is less severe than those previously reported (Cunnane and McAdoo 1987). We do, however, show the same changes in the fatty acid profile of the serum (L. Gambling et al., unpublished data) which suggests that, if the nutritional deprivation was to continue, we could result in the same pattern of changes.

In a recent paper, Brandao and colleagues suggested that the haemoglobin concentration of the red blood cell is important in maintaining flexibility (Brandao et al. 2009) of the membrane. They used optical tweezers to examine the properties of individual RBC from controls and patients with differing degrees of iron deficiency. They demonstrated a decrease in elasticity of the RBC, associated with a decrease in haemoglobin content of the cells. Why this should be the case is not clear, but is likely to be related to the architecture of the cell.

Why do the neonatal cell membranes behave differently from those of their mothers? Is it related to the level of iron deficiency? There are various studies which show that membrane rigidity is dependent on the level of iron deficiency, i.e. the more severe the iron deficiency, the more rigid the erythrocyte membrane (Yip et al. 1983; Anderson et al. 2000; Garcia et al. 2005). However, this does not explain our data, as the neonates born to iron-deficient mothers at both birth and day 10 had lower haematocrits,

therefore more iron deficient, than their mothers. Despite this, the sensitivity to osmotic stress is not changed.

Another possibility for the difference between the maternal and neonatal erythrocytes is that the adaptability of the neonates membranes to the stress of iron deficiency is greater than maternal erythrocytes. It has been well documented in the literature that erythrocytes from healthy human neonates differ from those of the adults. Neonatal erythrocytes are known to have increased osmotic resistance during the first 4–6 weeks of life in humans (Jain 1989), (Steiner and Gallagher 2007). Human neonate erythrocytes also show decreased deformability compared to their adult counterparts (Steiner and Gallagher 2007).

The final possible explanation for the difference in response between the mothers and neonates to osmotic pressure is a difference in membrane lipid composition. In general, the membrane lipid composition and cholesterol content are carefully regulated by the cell, but the proportions vary with the stage of the cell cycle and age (Clandinin et al. 1991). They are also likely to be affected by the exogenous fatty acids from the diet or by altered activities of lipid-metabolising enzymes, such as fatty acid desaturases (Clandinin et al. 1991; Ariyama et al. 2010). Various investigators have reported that the iron-deficient erythrocytes are more susceptible to oxidative stress (Tichelaar et al. 1997; Sanghani and Haldankar 2006). They have suggested that this may at least partly be due to impaired synthesis of lipid component of their membrane (Tichelaar et al. 1997). Our results do not support this hypothesis. There are only marginal differences in proportions of saturated and unsaturated fatty acids in the membranes of both the mothers and their neonates, probably not sufficient to account for the changes in properties.

In summary, the results presented in this paper show the hierarchy of importance of iron metabolism after birth. It not only helps in our understanding of the effect of iron deficiency during pregnancy, but also poses many questions.

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