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Effect of dietary copper deficiency on iron metabolism in the pregnant rat

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Cu and Fe metabolism are known to be linked, but the interactions during pregnancy are less well studied. In the present study we used rats to examine the effect of Cu deficiency during pregnancy on Fe and Cu levels in maternal and fetal tissue and on the gene expression profile of proteins involved in Cu and Fe metabolism in the placenta. Rats were fed diets with different Cu contents before and during pregnancy. Samples were collected on day 21 of gestation. Cu levels, ceruloplasmin activity and serum Fe all decreased in maternal serum of Cu-deficient animals. Maternal liver Fe inversely correlated with liver Cu. Placental Cu levels decreased with no change in Fe. Fe and Cu levels both decreased in the fetal liver. The drop in maternal liver Cu was significantly correlated with a decrease in organ weight of fetal liver, lung and kidney. No changes were observed in mRNA expression of Cu transporter 1, Menkes P-type Cu-ATPase 7A, Wilson P-type Cu-ATPase 7B, cytochrome-c oxidase, and Cu chaperone Atox1 in the placenta of Cu-deficient dams. Transferrin receptor 1 and the Fe-responsive element (IRE)-regulated divalent metal transporter 1 (DMT1) were up regulated; while ferroportin and non-IRE1-regulated DMT1 levels did not change. These data show that Cu deficiency during pregnancy not only has a direct effect on Fe levels but also regulates the expression of Fe transporters. The pattern closely mirrors that seen in Fe deficiency, suggesting that the changes are a consequence of the decrease in serum Fe, implying that the developing fetus not only suffers from Cu, but also from Fe deficiency.

Placenta: Copper deficiency: Iron metabolism: Fetal programming

The metabolism of Fe and Cu are closely linked. Initial observations were made in 1927, where Fe salts failed to cure anaemia in rats, while administration of Cu in the form of ashed foodstuff restored their Hb levels (Waddell *et al.*, 1927). Since then, several studies have confirmed the relationship. In general, severe Cu deficiency causes changes in Fe metabolism leading to anaemia and liver Fe accumulation. The mechanism involved is not fully understood, but is probably related to decreased expression and levels of enzymes such as ceruloplasmin (Cp) and hephaestin, which are multicopper oxidases required for Fe efflux. In support of this, mice with a mutation in hephaestin are anaemic and accumulate Fe in the gut (Vulpe *et al.*, 1999) and patients with aceruloplasminaemia suffer from anaemia rather than Cu deficiency (Harris *et al.*, 1995, 1998). Danzeisen *et al.* (2002) discovered a similar oxidase in a placenta cell line (BeWo cells) which is regulated by both Cu and Fe status.

We know that the dietary intake of Cu in women aged 19–24 years is only 78 % of the recommended daily intake (0·9–1·2 mg Cu/d; IOM, 2001). During pregnancy this may be even more of a problem as the need of micronutrients increases due to the requirements of a growing fetus. Cu is essential for fetal development and maternal dietary Cu deficiency can have both short- and long-term consequences (for a review, see Keen *et al.*, 2003). The extent to which Cu deficiency affects pregnancy outcome is very much dependent on the degree of Cu limitation. Severe Cu deficiency can lead to reproductive failure, early embryonic death and gross structural abnormalities in the fetuses (Prohaska & Brokate, 2002). In contrast, moderate or mild Cu deficiency has little effect on either the number of live births and neonatal weight (Masters *et al.*, 1983). However, the fetuses can suffer from lipid deposits in the connective tissue, cardiac haemorrhage, and skeletal defects (Wildman *et al.*, 1995). It is therefore important to characterise the effect that Cu deficiency during pregnancy has on Fe metabolism and to elucidate the mechanism behind such alteration.

During pregnancy the transport of micronutrients from the mother to the fetus takes place across the placenta. The mechanism for Cu transport across the placenta to the fetus has not been clearly elucidated. It is assumed that Cu transporter 1 (Ctr1), a Cu transporter in the plasma membrane, is responsible for the translocation across the apical membrane (Zhou & Gitschier, 1997). Virtually nothing is known about the transport mechanism for Cu once it is inside the placenta cells. Presumably it involves the same Cu chaperones as described for other tissues (for a recent review, see Prohaska & Gybina, 2004). One of these Cu chaperones, Atox1, binds Cu and carries Cu to either Menkes P-type Cu-ATPase 7A (ATP7A) or Wilson P-type Cu-ATPase 7B (ATP7B),

Abbreviations: ATP7A, Menkes P-type Cu-ATPase 7A; ATP7B, Wilson P-type Cu-ATPase 7B; Cp, ceruloplasmin; Ctr1, Cu transporter 1; DMT1, divalent metal transporter 1; IRE, Fe-responsive element.

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depending on the tissue. In the placenta, both ATP7A and ATP7B are expressed, and their specific locations may be relevant to the paths followed by Cu through the placenta (Hardman *et al.*, 2004).

The uptake of Fe into the placenta is through transferrin receptor-mediated endocytosis. In species with a haemochorial placenta such as rodents and man, Fe attached to transferrin binds to transferrin receptor on the brush-border membrane of the placenta where it is internalised in endosomes (McArdle *et al.*, 1985*a*,*b*). The endosomes are acidified and the Fe released. It moves into the cytoplasm, presumably via the divalent metal transporter 1 (DMT1), and is carried across the placenta and into fetal circulation. The efflux is probably through Fe-regulated gene 1 (ferroportin) and the Fe^{2+} is oxidised to Fe^{3+} before incorporation into fetal transferrin by a ferroxidase similar, but not identical, to Cp (Danzeisen *et al.*, 2002). The different genes respond differently to Fe deficiency. There is an increase in expression of transferrin receptor 1 and the Fe-responsive element (IRE)-regulated form of DMT1. In contrast, the non-IRE-regulated form of DMT1 and ferroportin show no changes in expression levels (Gambling *et al.*, 2001).

Present data on the effect of mild Cu deficiency on maternal to fetal transport of Fe are contradictory. A study by Wapnir *et al*. (1996) showed that fetal liver Fe levels increased when exposed to Cu-deficient diets, whereas another study reported a decrease (Ebesh *et al.*, 1999).

The purpose of the present study was to determine the effect of mild Cu deficiency on maternal and fetal Cu and Fe metabolism, growth and development. In order to understand the mechanism for any changes in fetal Cu and Fe metabolism we examined whether there was an altered expression of genes encoding the proteins of Cu and Fe transport in the placenta.

Methods

Experimental diets

The diets were based on dried egg albumin and conformed to American Institute of Nutrition guidelines for laboratory animals (American Institute of Nutrition, 1980). $CuSO_4$ was added to achieve levels of added Cu of 5 (control diet), or 2·5 (50 % control Cu) and 0·75 mg Cu/kg (15 % control Cu) of diet. The diets were identical in all other respects. Fe concentration was held at 50 μg Fe/kg. Dietary ingredients were purchased from Mayjex Ltd (Chalfont St Peter, Bucks, UK), BDH Chemicals (Poole, Dorset, UK) or Sigma (Poole, Dorset, UK).

Experimental animals

All experimental procedures were approved by the Home Office and the Ethics Committee at the Rowett Research Institute and conducted in accordance with the UK animals (Scientific Procedures) Act, 1986. Experiments were performed using weanling female rats of the Rowett Hooded Lister strain, bred at the Rowett Research Institute. They were grouped housed in cages, under a 12 h light–dark cycle and with constant temperature and humidity. All animals were fed *ad libitum* and provided with distilled water.

Twenty-four female weanling rats were fed the control diet for 4 weeks, before being randomly assigned to one of the three diet treatments (eight rats per treatment), control or Cu-deficient diet having 50 and 15 % Cu levels compared with the control for 4 weeks before mating.

Tissue samples

On day 21 of gestation, the dams were killed by stunning and cervical dislocation. The numbers of fetuses and placentas were counted, and the number of resorption sites observed in the uterus was recorded. Placentas associated with healthy fetuses were weighed and frozen in liquid N_2 before being stored at −80°C. Fetal livers, hearts, kidneys, and lungs were collected from six fetuses, chosen from each mother at random, rapidly dissected, weighed and frozen in liquid N_2. Livers, kidneys, spleen, hearts, lungs, pancreas, ovary and adrenals were dissected from all dams, weighed and frozen in liquid N_2 before being stored at −80°C. Maternal blood was collected in non-heparinised tubes and centrifuged at 1000 ***g***, at 4°C, for 10 min. The resulting serum was stored in metal-free Eppendorf tubes (Axygen Scientific, Union City, CA, USA) at −80°C.

Atomic absorption spectrophotometric analysis

Fe and Cu levels in maternal serum and in tissue from dams and fetuses were measured by graphite furnace atomic absorption spectroscopy (AAnalyser6600; Perkin Elmer, Norwalk, CT, USA) according to standard procedures, as previously described (Gambling *et al.*, 2001). To differentiate between haeme and non-haeme Fe in the serum and tissue, samples were treated with 20 % (w/v) TCA, heated to 96°C for 5 min and the supernatant fraction collected as described previously (Gambling *et al.*, 2002). Quality controls were included as necessary. Repeat measurements gave variance less than 15 %. Standards were obtained from BDH Laboratory Supplies (Poole, Dorset, UK). The laboratories are certified to ISO 9001.

Real-time quantitative reverse transcription polymerase chain reaction

Complementary DNA PCR primers for the rat were designed using Primer Express (version 1.5; Applied Biosystems, Foster City, CA, USA) software from DNA sequences obtained from GenBank (Table 1). All primer sets had a calculated annealing temperature of 58°C. Primers were synthesised by MWG Biotech (Ebersberg, Germany).

Total RNA was isolated from tissue following the method of Chomczynski & Sacchi (1987) and treated with DNAse I (Boehringer Mannheim, Mannheim, Germany). First strand complementary DNA was synthesised by priming with hexamers using the Taqman RT Reagent Kit (Applied Biosystems). Reverse transcription was performed in 20 μl reactions using 200 ng of DNAse-treated RNA.

Real-time PCR amplification and analysis was performed using a 7700 Sequence Detection System (Applied Biosystems) and ABI prism software (version 1.9; Applied Biosystems). Reactions were performed in 25 μl volume with 300 nM primers and 5 μl cDNA. $MgCl_2$, nucleotides, buffer

Table 1. Description of the primers used for real-time reverse transcription polymerase chain reaction analysis

Gene name	Species	GenBank accession no.	Forward primer*	Reverse primer*	Fragment (bp)
Transferrin receptor 1	Rat	M58040	1757–1779	1818–1838	82
DMT1	Rat	AF008439	2168–2188	2228–2247	80
DMT1 (non-IRE)	Rat	AF029757	1650–1673	1700–1722	73
Ferroportin	Rat	AF394785	1549–1573	1606–1626	78
Ctr1	Rat	AF268030	17–34	76–96	80
ATP7A	Rat	NM052803	1996–2021	2055–2082	87
ATP7B	Rat	NM012511	1766–1786	1815–1837	72
Cu chaperone Atox1	Rat	NM_053359	99–116	180–200	102
18S2	Rat	V01270	941–959	986–1006	66

DMT1, divalent metal transporter 1; IRE, Fe-responsive element; ATP7A, Menkes P-type Cu-ATPase 7A; ATP7B, Wilson P-type Cu-ATPase 7B.
* Coordinates according to the GenBank DNA sequence.

and Taq DNA polymerase were included in the SYBR Green Master Mix (Applied Biosystems). Amplification specificity was assessed by agarose gel electrophoreses.

Standard curves were generated from increasing amount of cDNA made from total rat placenta control RNA. The C_T values were used to calculate and plot a linear regression line by plotting the logarithm of template concentration (x-axis) against the C_T value (y-axis). These regression lines were used to calculate the expression level (ng total RNA) for unknown samples. Data were normalised to the expression levels of 18 S. For comparison between groups, controls were taken as 100 %.

Ceruloplasmin assay

Maternal serum and placenta tissue from the control and 15 % Cu − groups were analysed for Cp oxidase activity. The placentas were homogenised in 0·02 M-N′-(2-hydroxyethyl)piperazine-N-2-ethanesulfonic acid, 0·25 M-sucrose buffer with a homogeniser (Ultra Turrax T25; IKA Labortechnik, Stauful, Germany). The homogenates were fractionated into a membrane and soluble fraction by centrifugation at 50 000 rpm (108 000 ***g***) at 4°C for 1 h. Cp oxidase activity (ferroxidase activity) was determined by measuring its *p*-phenylenediamine oxidase activity in the presence and absence of 150 mM-sodium azide (Sunderman *et al.*, 1984; Gambling *et al.*, 2004). Briefly, the samples were incubated in 200 mM-acetate buffer, pH 5·5, at 37°C for 1 h and read at 560 nm (ELx800 UV plate reader; Bio-Tek Instruments, Inc., Winooski, VT, USA). Human Cp (Vital Products, Boynton Beach, FL, USA) was used as a standard. Azide-sensitive ferroxidase activity was expressed as μg Cp/mg protein.

Statistics

For each dam, fetal data were averaged and the data recorded as a single point. All results are presented as mean values with their standard errors. A minimum of six measurements for each diet group was used for analysis. The effect of diet was assessed by one-way ANOVA and, as appropriate, either statistically or biologically, treatment means were also compared with a *post hoc t* test. Linear regression was used to look for relationships between Cu and Fe levels. Relationships between organ weight and body weight were investigated using allometric analysis (linear regression of (natural) log (organ weight) on log (body weight)). This approach was extended to investigate relationships between fetal organ weight, litter size, and maternal liver Cu concentrations by means of multiple linear regressions using (natural) log-transformed data.

For real-time PCR the change in expression between mRNA levels of the control group and the 50 and 15 % Cu-deficient groups were presented as the ratio of 18 S expressions in each particular sample. Each group contained amplified cDNA from five to eight animals, and the average of these measurements was used to calculate mean and SEM. Significant differences between treated and control groups were determined using one-way ANOVA. Statistical analyses were performed in GenStat (8th edition, release 8.1; VSN International Ltd, Hemel Hempstead, Herts, UK).

Results

There was no effect of dietary treatment on body-weight gain of the dams, number of fetuses, resorption sites or packed cell volume (Table 2). In addition, no significant changes were observed for any of the maternal organs examined (based on allometry analysis with regression of log (organ weight) on log (body weight) + diet; data not shown).

A significant ($P<0·001$), diet-induced, decrease in maternal serum Cu levels was observed (Fig. 1(A)), together with a parallel decrease in Fe levels (Fig. 1(B); $P=0·020$; r^2 0·35).

Maternal liver Cu levels were significantly ($P<0·001$; r^2 0·59) decreased as levels in the diet were reduced (Fig. 2(A)). When comparing the dietary extremes of the control and the 15 % Cu, the maternal liver Fe level significantly increased ($P=0·033$; two-tailed Mann–Whitney t test) (Fig. 2(B)).

Between the two extremes of dietary Cu, 5 mg/kg (control) and 0·75 mg/kg (15 % of control) Cu oxidase activity was significantly reduced in maternal serum and in the soluble fraction of the placenta (presumably representing serum contamination of the soluble fraction) (Table 3). Cu oxidase activity in the membrane fraction of the placenta did not change.

In other tissues, we measured both total and non-haeme Fe. Overall, the changes in maternal Cu status were reflected by alterations in both placental and fetal parameters. When comparing the dietary extremes of the control and the 15 % Cu, the placental Cu levels decreased ($P=0·043$; two-tailed Mann–Whitney t test) (Fig. 2(C)) while there were no changes in either placental non-haeme (not shown) or total Fe (Fig. 2(D)). The fetal liver Cu ($P=0·003$; r^2 0·40)

Table 2. Effect of maternal copper deficiency in the rat on growth and fertility
(Mean values with their standard errors; six to eight rats per group)

	Cu level					
	5 mg/kg		2·5 mg/kg		0·75 mg/kg	
Diet group	Mean	SEM	Mean	SEM	Mean	SEM
Maternal body weight (g)	320·73	3·95	319·83	9·05	334·82	5·76
Total no. of fetuses (n/per dam)	14·43	0·35	11·67	1·98	13·67	1·09
Resorption sites (*n*/dam)	0·29	0·17	1·17	0·48	1·00	0·63
Packed cell volume (%)	40·71	0·91	40·20	1·16	38·83	0·70

and non-haeme Fe levels ($P<0·001$; r^2 0·47) were significantly correlated to maternal Cu status (Figs. 3(A) and (B)).

Data regarding the influence of maternal Cu deficiency on fetal development can be analysed in two ways: related to maternal dietary Cu intake or related to maternal liver Cu levels. We consider the latter approach more appropriate as it provides a better indicator for Cu status and have investigated the influence of maternal liver Cu concentration on fetal weight and organ weights, allowing for a correction for litter size (Fig. 4). There was a tendency to a reduced fetal weight with low maternal liver Cu levels ($P=0·053$; data not shown). Maternal liver Cu levels were positively correlated to the organ weight of fetal liver ($P=0·023$; r^2 0·26), lung ($P=0·022$; r^2 0·26) and kidney ($P=0·040$; r^2 0·21), which was lower for low maternal liver Cu concentration (Fig. 4). There was no alteration in relative heart size ($P>0·1$; data not shown).

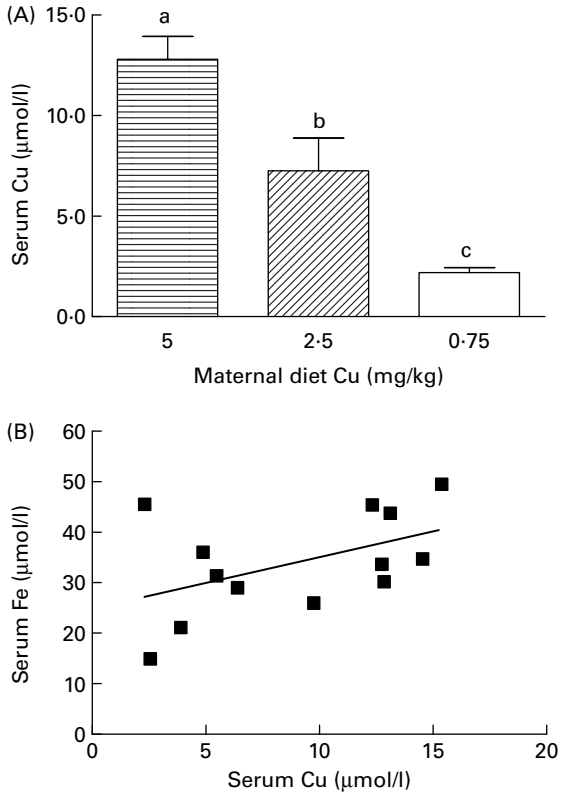

Fig. 1. (A) Maternal serum Cu concentration in pregnant female rats according to the Cu content of the maternal diet (eight rats per diet group). Values are means, with their standard errors represented by vertical bars. [a,b,c] Mean values with unlike letters were significantly different ($P<0·001$; ANOVA). (B) Relationship between maternal serum Cu and Fe concentration in pregnant female rats (r^2 0·35; $P=0·020$).

Examination of genes involved in Cu and Fe metabolism revealed a change in expression levels of specifically genes involved in Fe metabolism. In the placenta, mRNA levels of transferrin receptor 1 were significantly increased ($P=0·0014$; r^2 0·48) with decreasing maternal liver Cu levels (Fig. 5(A)).

The expression of the two DMT1 transcripts, the Fe-responsive form (IRE)-regulated and non-IRE-regulated showed different patterns in the maternal placenta. The IRE-regulated transcript increased ($P=0·013$; r^2 0·33) with decreasing levels of Cu (Fig. 5(B)), while the non-IRE-regulated form of DMT1 did not change ($P=0·39$) (Fig. 5(C)). Ferroportin levels did not change significantly and there were no significant changes in the mRNA level of any of the Cu proteins examined, ATP7A and B, Ctr1, or Cu,Zn superoxide dismutase 1 (data not shown).

Discussion

The aim of the present study was to determine the effect of mild dietary Cu deficiency during pregnancy in rats on maternal growth, on pregnancy outcome and on Cu and Fe status of both the mothers and their fetuses. The data show that the severity of Cu deficiency had no effect on maternal growth and number of fetuses. The present results confirm previous data showing that Cu deficiency during pregnancy in the rat does not affect maternal outcome (Ebesh *et al.*, 1999).

Interestingly, we found that maternal liver Cu status affected fetal organ weight, and also tended to affect fetal body weight, with fetal liver, lung and kidney being reduced in weight as maternal liver Cu levels decreased. Allometry analyses indicated strong significant ($P<0·001$; data not shown) linear relationships between the log-transformed organ weights and log-transformed fetal weight. In a previous study, no effect was seen in fetal organ weight (Ebesh *et al.*, 1999). One likely cause of the difference between the present study and the one by Ebesh *et al.* (1999) is that the latter fed rats a Cu-deficient diet (0·54–0·58 mg Cu/kg diet) only from gestational day 10 until delivery. Our protocol gave the dams a Cu-deficient diet for 7 weeks by the end of gestation. Data from the present study and from a recent publication (Andersen *et al.* 2006) suggest that growth might be regulated earlier on in pregnancy where major organ development occurs – a period during development not covered in

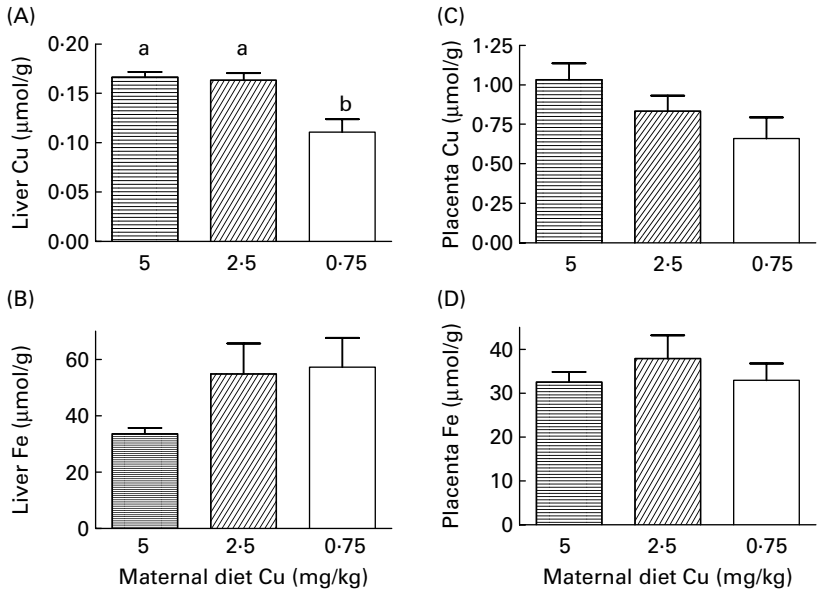

Fig. 2. Maternal liver Cu (A), liver Fe (B), placenta Cu (C) and placenta Fe according to the Cu content of the maternal diet (eight rats per diet group). Values are means, with their standard errors represented by vertical bars. [a,b] Mean values with unlike letters were significantly different (P=0·033; ANOVA).

the study by Ebesh *et al*. (1999). However, it should also be pointed out that the relationship between size and Cu content in the present study is dependent on correcting for litter size and, as will be apparent from Fig. 4, one particular data point at a maternal Cu content of about 2 μmol/g. We have no reason to exclude this point, but it does mean the present results should be treated with some caution.

Our model of mild maternal Cu deficiency causes significant changes in hepatic Cu and Fe levels, but maternal packed cell volume remains unchanged, suggesting only a mild state of Cu deficiency. Although placenta Fe levels appeared not to be affected by the reduced dietary intake of Cu, fetal liver Fe levels were reduced. Placental Fe would not be expected to change. It acts as a conduit for Fe. As such, the levels of Fe in the liver are a better indicator of transport activity than levels in the placenta itself. The effects of Cu on Fe metabolism were opposite in the mother and the fetus; maternal liver Fe increased, whereas fetal liver Fe content decreased. Our data show that the primary consequence of low dietary Cu is a compromised maternal to fetal transport of Cu and Fe. This finding is in agreement with previously published data by Ebesh *et al*. (1999).

The expression data in the placenta suggest that it is responding in the same way as it does to a directly induced Fe deficiency. We have previously shown that maternal Fe deficiency results in increased expression of transferrin receptor 1 and DMT1 and no change in ferroportin expression. In the present study we obtained the same results. The data also give some intriguing insights into the possible regulation of Fe transport. Hepcidin

Table 3. Ceruloplasmin (Cp) activity in placenta and serum of gestational day 21 copper-deficient rats

(Mean values with their standard errors of the mean; three to eight rats per group)

Diet group	Cu level			
	5 mg/kg		0·75 mg/kg	
	Mean	SEM	Mean	SEM
Placenta soluble fraction (μg Cp/g protein)	4·29	0·79	1·62*	0·23
Placenta membrane fraction (μg Cp/g protein)	11·02	0·73	11·07	1·18
Maternal serum (μg Cp/g protein)	11 186·1	1082	6639·2*	516

* Mean value was significantly different from that of the 5 mg/kg group (P<0·05).

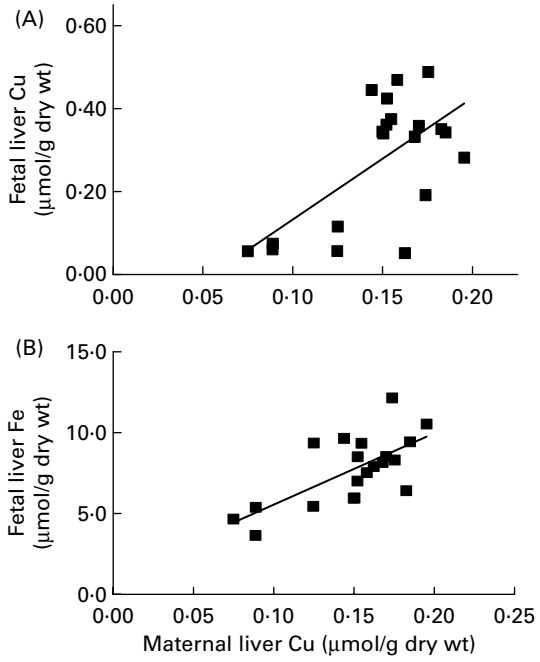

Fig. 3. (A) Relationship between maternal liver Cu and fetal liver Cu (r^2 0·40; P=0·003). (B) Relationship between maternal liver Cu and fetal liver non-haeme Fe (r^2 0·47; P<0·001).

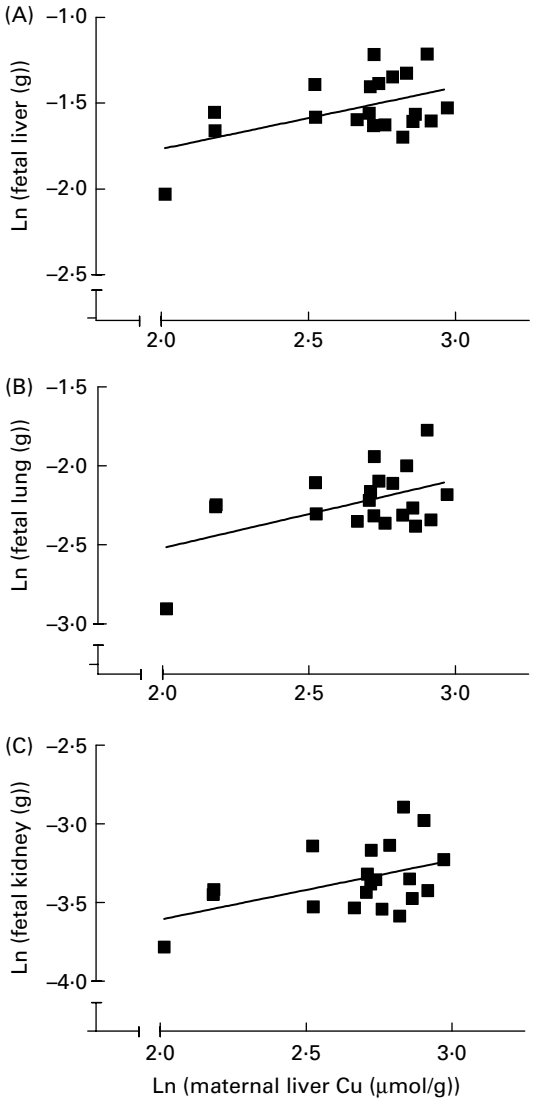

Fig. 4. (A) Relationship between maternal liver Cu and Ln fetal liver weight (r^2 0·26; P=0·023). (B) Relationship between maternal liver Cu and Ln fetal lung weight (r^2 0·26; P=0·022). (C) Relationship between maternal liver Cu and Ln fetal kidney weight (r^2 0·21; P=0·040).

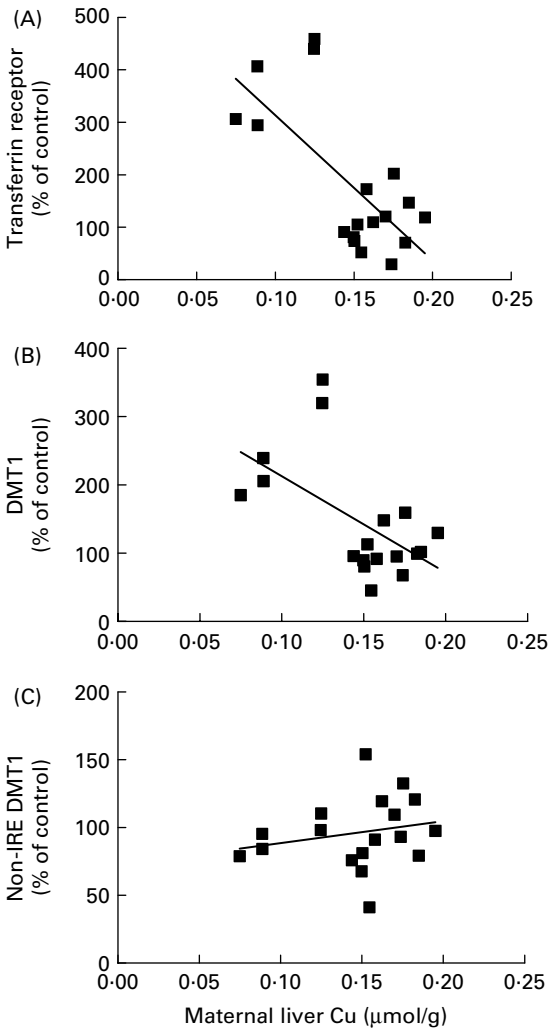

Fig. 5. (A) Relationship between maternal liver Cu and placenta transferrin receptor mRNA (r^2 0·48; P=0·0014). (B) Relationship between maternal liver Cu and placenta divalent metal transporter 1 (DMT1) (r^2 0·33; P=0·013). (C) Relationship between maternal liver Cu and placenta non-Fe-responsive element (IRE) DMT1 (P=0·39).

is a small peptide thought to be involved in the regulation of Fe absorption and in the regulation of systemic Fe metabolism. Its expression in the liver is proportional to Fe requirements (Mazur *et al.*, 2003). Given the increase in maternal liver Fe, therefore, one would expect an increase in hepcidin expression. This would result in a decrease in expression of transporters in the gut and, if maternal hepcidin regulated placental transport, the placenta. This is clearly not the case, as the expression of the placenta transporters increases, so we can conclude that maternal hepcidin is not involved in regulation of transplacental Fe transfer.

In contrast to Fe, regulation of Cu transport seems to occur primarily by altered location of the transport proteins. The mechanism for Cu transport across the placenta to the fetus has not been clearly elucidated. It is assumed that Ctr1, a Cu transporter in the plasma membrane, is responsible for the translocation across the apical membrane (Zhou & Gitschier, 1997). Ctr1 is expressed in the mammalian placenta (Gambling *et al.*, 2004; Hardman *et al.*, 2006). The regulation of Ctr1 is tissue specific and dependent on Cu status (Bauerly *et al.*, 2005; Kuo *et al.*, 2006). The data presented here showed no changes in expression of Ctr1 mRNA levels in the placenta. The absence of changes in Ctr1 mRNA levels by dietary Cu deficiency is not unexpected. Studies in postnatal Cu-deficient rats showed no changes at the mRNA level in liver, intestine and hypothalamus (Lee *et al.*, 2000; Kuo *et al.*, 2006) and a recent study using a placenta cell line (Jeg-3 cells) showed no change in Ctr1 protein level in cells cultured in media with increased or decreased Cu conditions (Hardman *et al.*, 2006). One important difference that must be taken into account when considering Cu or Fe status in the placenta *in vivo* in comparison with *in vitro* systems is that the placenta has a fetus attached, and hence fetal levels need to be considered for indicators of placental status. So, for example, it is not entirely surprising the Cu,Zn superoxide dismutase 1 levels are not changed, because the steady-state levels of Cu

in the placenta are not significantly changed, but the total amount transferred by the tissue are significantly reduced.

Virtually nothing is known about the transport mechanism for Cu once it is inside the placental cell. Presumably it involves the same Cu chaperones as described for other tissues (for a review, see Prohaska & Gybina, 2004). Atox1 is a small Cu chaperone which has an essential role in delivering Cu to the Cu-ATPase ATP7A (Hamza *et al.*, 2001). The Menkes (ATP7A) and Wilson (ATP7B) proteins are Cu-transporting ATPases that are defective in human Cu-deficiency disorders. The two proteins have different cellular localisation and it is possible that they have different functions in the placenta (Muramatsu *et al.*, 1998; Hardman *et al.*, 2004). The data presented here showed no changes in expression of Atox1, ATP7A or ATP7B mRNA levels in the placenta. The absence of changes in mRNA levels was not unexpected, as the two Cu-ATPases have been shown to be subjected to post-translational modification rather than transcriptional regulation by Cu (Paynter *et al.*, 1994; Mercer & Llanos, 2003).

Our data emphasise the importance of a sufficient intake of micronutrients, especially Cu and Fe, during pregnancy for the normal development of the baby. Deficiency in one micronutrient can lead to imbalances in nutritional status in others, with potentially serious consequences both in the short and in the long term for the offspring. We have shown, for example, that maternal Fe deficiency results in hypertension in the offspring, and the present data would imply a similar outcome for Cu deficiency. It becomes more important, therefore, to develop an accurate and specific suite of markers for the assessment of Cu status in women of childbearing age and especially in pregnancy.

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