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EARNEST

EARly Nutrition programming- long term follow up of Efficacy and Safety Trials and integrated epidemiological, genetic, animal, consumer and economic research

Instrument: Integrated Project

Thematic Priority 5.4.3.1: Food Quality and Safety

Final public report on activity3.3.1:

Maternal iron deficiency and post-natal blood pressure

Period covered from 15.04.2005 to 14.10.2010

Start date of project: 15.04.2005 Duration: 5,5 Years

Organisation Name of Lead Contractor for this report: RRI

ACTIVITY OBJECTIVES AND MAIN TASKS

Iron deficiency anaemia is a common nutritional deficiency during pregnancy, with serious consequences, increased risk of neonatal morbidity, mortality, developmental delay, and an increased risk of cardiovascular disease in adult offspring. Over the last several years we have developed a rodent model of maternal iron deficiency that mimics many of the problems observed in humans, in particular hypertension. This model has been used to study how maternal iron deficiency affects the growth and development of the offspring. The objective of this activity is to investigate possible mechanisms underlying the hypertension in the offspring of iron deficient mothers. We tested the hypothesis that the cause of the hypertension is associated with gene and protein expression in known to be involved in the generation of blood pressure. The possibility of there being critical windows in fetal development where the programming of hypertension occurs was also investigated. This study has concentrated on the kidney in the first instance, and latterly yolk sac and heart. DNA array and proteomic techniques were used to investigate the changes in gene and protein expression between offspring of control and Fe deficient pregnancies in the target tissue. Expression of target genes and proteins was characterised. Possible pathways, genes and gene products perturbed by maternal Fe deficiency were identified in these experiments.

RESULTS

Blood pressure of offspring Fe deficient during first half of gestation

(Deliverables 36)

Previously our group have published data from post-implantation embryo culture experiments that iron status over a 2 day period in gestation, 10.5 to 12.5 days, is important for optimal vascular development of the yolk sac. In further experiments, we tested whether the window of deprivation had an effect on parameters at birth. Using our rat model of maternal iron deficiency during pregnancy, have indicated that maternal iron status during the first half of gestation is critically important in determining birth weight at the end of term. However, we did not show whether these changes were reflected in hypertension. This deliverable was designed to answer that question.

These experiments involved four groups of experimental animals; Group 1, rats fed control diet throughout pregnancy, Group 2, rats fed iron deficient diet throughout pregnancy, Group 3, rats fed iron supplemented diet during the first half of pregnancy only and and Group 4, rats fed iron supplemented diet during the second half of pregnancy only. The male offspring of these four groups were then cross-fostered to stock fed dams within 24 hrs of birth. Their blood pressure was measured at 44 weeks of age.

When the blood pressure data was analysed it fell into three, not four separate groups. Blood pressure in offspring whose mothers were deficient throughout pregnancy show the highest blood pressure. However those who were deficient for only part of pregnancy their blood pressures are the same. Their values intermediate between the control and deficient groups. The blood pressure data also show a strong correlation to both maternal (Fig 1A) and fetal hemtocrits, and fetal iron status (Fig 1B) when measured directly, as iron content of the liver, at day 21.5 of gestation. This data continues to verify the link between iron status during pregnancy and blood pressure in the offspring as adults, it also raises some questions. Why do the time-dependent effects, e.g. birth weight not continue through blood pressure? The implication is that either vascularisation in the early stages of gestation are not important in blood pressure later, or that the process can be reversed by giving sufficient iron later in gestation. This is clearly important information since it suggests that supplementation with iron in the last trimester may be of value in mitigating the effects of deficiency earlier in gestation.

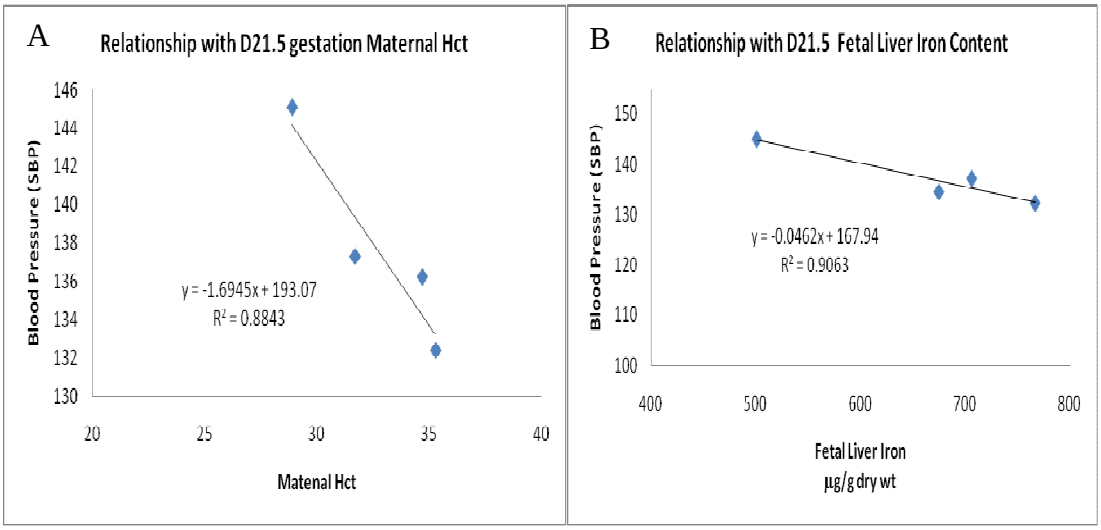

Figure 1 Offspring blood pressure at 44 weeks of age.
Plotted against (A) maternal hematocrit (B) fetal liver iron content

The role of the kidney in the induction of hypertension in offspring of iron deficient mothers

(Deliverables 37, 40, 63, 64)

The kidney plays an important function in long term blood pressure regulation. Interestingly it has previously been shown in our rodent model of maternal iron deficiency that fetuses and neonates of mothers fed an iron deficient diet during pregnancy have smaller kidneys at birth compared to controls. Therefore this activity aimed to investigate whether the altered kidney development or function underlay the hypertension seen in offspring of iron deficient mothers. Using western blotting, RT-PCR and enzyme activity assays the most common kidney derived causes of hypertension, such as nephron number, renin angiotensin system and renal sodium transport were investigated. Decreased renal cell number and impaired vasculogenesis were hypothesised to be responsible for the reduced kidney size, but there were no significant differences in the gene expression of markers of vasculogenesis/angiogenesis, and regulators of apoptosis and cell proliferation. This activity also dismissed the hypotheses of reduced nephron number and impaired sodium transport as possible causes of hypertension in offspring of iron deficient mothers. The renin angiotensin system showed significant increases in the renal renin mRNA expression, and pulmonary ACE mRNA and activity levels in newborn offspring of iron deficient mothers. However, these changes were temporary and disappeared within two weeks of birth.

Having checked and dismissed the involvement of common mechanisms behind hypertension, new hypotheses for possible mechanisms were generated based on the results of the DNA microarrays of the fetal kidney at D21.5 gestation. Through the EARNEST collaboration with NuGo (WP 3.5) we were able to carry out extensive analysis using Affimetrix rat whole genome arrays. Only 40% of the genes present on the whole genome array were expressed in the kidney. Of those genes only 6 % were regulated by maternal iron deficiency, 483 up-regulated and 359 down regulated. Analysis was initially restricted to genes with translated products of known function. Those with the highest fold change were S100 calcium binding proteins A8 and A9. These proteins are potent chemoattractants for monocytes. The genes with the largest down regulation were those involved in cation transport. Secondly, the DNA microarray study identified a group of genes directly involved in the collagen cross-linking including: procollagen, type V, alpha 1, lysyl oxidase, lysyl oxidase like 1, and procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 which were differentially regulated in the kidneys of offspring Fe-deficient mothers.

More in-depth analysis of the data set is ongoing using pathway analysis programmes such as Bibliosphere™ and Metacore™ Genomatix's Bibliosphere™ programme is a text-mining programme which identifies possible relationships between biological entities. The identities of the 842 differentially expressed genes were uploaded to this programme. The pathway map generated showed two nodes of particular interest. The centres of the nodes were identified as the transcription factor c-myc and the previously identified calcium binding protein S1008a. The analysis programme Metacore™ facilitates the identification of pathways and processes affected by maternal iron deficiency. Initial analysis indicates that in the kidney, 50 gene ontology processes have been significantly affected by maternal Fe deficiency. The leading ten processes are shown in Fig 2.

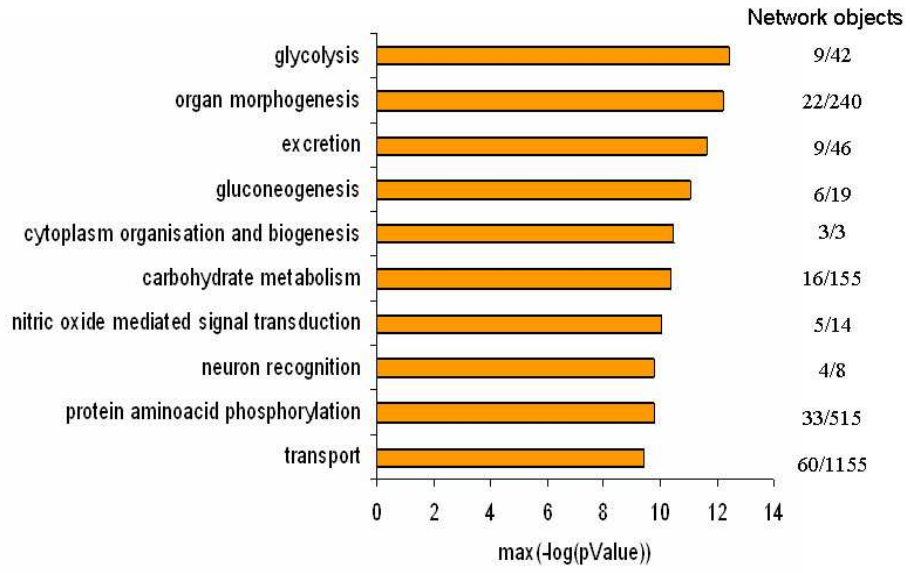

Figure 2 Top 10 gene ontology processes affected in the kidney

The results of the DNA microarray experiment suggested that gene and protein expression changes during development may result in altered kidney structure, with renal inflammation and altered collagen cross-linking, as possible causes of hypertension in the offspring of iron deficient mothers. Therefore further analysis was carried out on the kidneys from control and iron deficient mothers to investigate further this possibility.

The S100A8 and A9 proteins are mainly expressed by neutropihls and monocytes, and increased monocytes in the iron deficient kidney could attract macrophages resulting in inflammation. Random sections from control and iron deficient kidneys sampled at D21.5 of gestation, day of birth and 14 days postnatal, were stained with ED1 a macrophage marker. The results indicated that there were significantly less macrophages in the kidneys of iron deficient offspring when compared to those of controls. This indicates that whatever the role of the calcium binding proteins S100A8 and A9, they are not responsible for any macrophage infiltration, and inflammation in the developing kidney is unlikely to play a causative role in the hypertension seen in the offspring of the iron deficient mothers.

The possibility that Fe-deficiency induced alterations in collagen metabolism would lead to microvascular remodelling was investigated. Collagen and elastin are major components of the blood vessels determining their biochemical properties. Collagen content in kidneys and lungs was quantified in relation to hydroxyproline level (amino acid marker for collagen proteins) in the tissue samples and, collagen cross-links were analysed using an HPLC method. Maternal iron deficiency during pregnancy had no effect on the kidneys and lungs collagen content in 14 day (PN14) and 6 week old offspring. In the kidneys of PN14 offspring the level of unreducible cross-link, pyridinoline (Pyd) was not significantly different between the groups. The deoxypyridinoline (Dpd) content in kidneys at this time point was

below the detection level. The Pyd content in lungs was significantly reduced in the PN14 offspring of Fe-deficient mothers (P=0.04), but the Dpd level was not different between the groups. Whilst in 6 week animals, renal and pulmonary reducible cross-links: Pyd and Dpd, and nonreducible cross-links: HLNL and DHLNL were not significantly different between offspring of the control and Fe-deficient mothers (P=0.09 and P=0.08, respectively). Increasing the number of the animals per group up to 25 would make the differences significant.

Differential protein expression in fetal vascular tissue as a result of maternal Fe deficiency

(Deliverables 37, 40, 65)

Whilst the kidney is the first organ of interest when investigating hypertension the resistance provided by the heart and vascular tissue also have an impact. The yolk sac has been identified as the vascular tissue of most interest. This is due to data published by this group, and others, indicating that the first half of gestation is a 'critical window' for the programming effect. DNA microarray analysis was carried out as described above. In the yolk sac maternal iron deficiency leads to the differential expression of 768 genes. This is too large a number to be studied individually and pathway analysis was carried out in order to prioritise area further to study. By using Metacore^{TM} we are able to obtain lists of biochemical pathways, processes and networks which contain genes that have significant gene expression changes. These are ranked taking into account the number of genes changed in the pathway, the size of change and the significance value attributed to that change. When the list of pathways, processes and networks altered in the yolk sac by maternal iron deficiency was studied it became clear that the effects of micronutrient deficiency were far reaching. The pathways and processes affected were involved in the cell cycle and transcription regulation, through to the immune response and cytoskeletal organisation.

Altered gene expression in the hearts of offspring of iron deficient mothers

(Deliverables 37, 40, 65)

Differential protein expression has been studied in the offspring of iron deficient pregnancies at both fetal, day 21.5 of gestation, and postnataly at 6 weeks of age by 2D gels. Analysis of the fetal heart pools resulted in a total of 1179 spots. Of these 112 were significantly different between Control and Fe def groups at $p<0.05$, whilst 31 significant at $p<0.01$. Of the 31 significant at $p<0.01$, 11 were up-regulated in Fe def whilst 20 were down regulated. Putative protein identifications were possible for 26 spots ($p<0.01$), using LC-MS/MS and Mascot. Analysis of the postnatal hearts resulted in 680 spots, with 21 significantly different between controls and Fe def groups at $p<0.05$, whilst 7 were sig diff at $p<0.01$. At $p<0.01$ for postnatal hearts 2 were up-regulated and 5 down regulated. Putative protein identifications were possible for 15 spots in the post natal group ($p<0.05$). As with the DNA array analysis the protein lists can be further studied by either using straight forward comparison of the lists, or by pathway analysis. Unexpectedly there were no proteins differentially expressed in the fetal hearts that remained altered in hearts at 6 weeks of age. However using pathway analysis we were able not only to study the individual proteins, but the pathways such differential expression would affect. This analysis indicates that the differentially expressed proteins in the D21.5 and 6 week heart, although different, are part of the same biological pathways. Interestingly three out of the seven pathways identified as being susceptibly to iron deficiency involve muscle contraction.

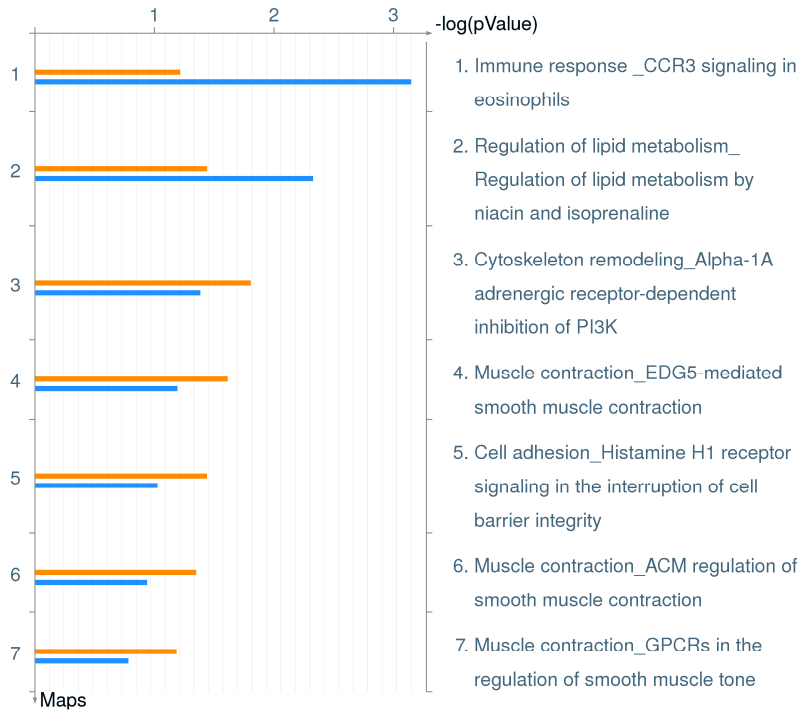

Fig 2. GeneGo Pathway maps significantly affected by maternal iron deficiency. D21.5 expression indicated by the blue bar and expression in the 6 week heart by the orange.

CONCLUSION

The data generated by this activity indicates that the involvement of one of the more common mechanism of blood regulation being responsible for the hypertension seen in the offspring of iron deficient mothers is unlikely. By using the less targeted approaches of DNA microarray analysis and proteomics a new hypothesis has been generated linking both the physiological and molecular findings. The common factor is the involvement of process, pathways and proteins involved in cell and organ structure. Changes which may result in increased vascular resistance, leading to increased blood pressure. This hypothesis will form the foundations for future research into the effect of maternal iron deficiency on offspring development and health.

Objectively verifiable indicators

Publications

Gambling et al (2009) 'Fetal iron status regulates maternal iron metabolism during pregnancy in the rat' Am J Physiol 296, R1063-70

McArdle et al (2008) 'Copper and iron transport across the placenta: Regulation and Interactions' J Neuroendo. 20, 427-31

McArdle et al (2006) 'Fetal Programming: Causes and Consequences as revealed by studies of dietary manipulation in rats' Placenta 27, S56-60

PhD Thesis

Alicja Czopek 'Effect of maternal iron deficiency during pregnancy on kidney development and blood pressure regulation in the rat offspring'. University of Aberdeen 2009 (U of A ref HHW2Y)

Meeting Abstracts

Gambling et al 'Interactions between iron and copper during development' Biochemical Society Focused Meeting, London, UK July 2008

Gambling et al ‘Fetal iron status regulates maternal iron metabolism during pregnancy in the rat’ Physiological Society Meeting, Edinburgh, UK February 2007
Gambling et al ‘Maternal iron deficiency during pregnancy in the rat induces high blood pressure, obesity and dyslipidaemia in her offspring’ Developmental Origins of Health and Disease Conference, Canada, November 2005
In addition, posters of the work were presented at the EARNEST meetings in Tarragona, Brussels and Prague.