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Serum concentrations of homocysteine are elevated during early pregnancy in rodent models of fetal programming

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Maternal malnutrition can lead to fetal abnormalities and increase susceptibility to disease in later life. Rat models have been developed to study the physiology and metabolism underlying this phenomenon. One particular model of 50 % protein restriction during pregnancy, the low-protein diet (LPD) supplemented with methionine, has been developed to investigate the underlying mechanisms. Recent studies have shown that rats fed a LPD during only the first 4 d of pregnancy produce offspring that develop hypertension. These results suggest that the very earliest stages of embryo development are susceptible to diet-induced heritable changes. We demonstrate a marked elevation of maternal serum homocysteine (hcy) concentrations during the initial phases of pregnancy in both rats and mice fed an LPD. Fetal growth and many of the circulating amino acids are similarly perturbed in both rats and mice fed the LPD during pregnancy, indicating that the response to the LPD diet is similar in rats and mice. These findings allow us to exploit the advantages of the mouse experimental system in future analyses aimed at understanding the molecular basis of fetal programming. Our present findings are discussed with particular reference to mechanisms which may initiate fetal programming, and to the feasibility of dietary interventions aimed at reducing early pregnancy loss and pre-eclampsia in man.

Preimplantation mouse embryos: Maternal malnutrition: Methylation: Early pregnancy loss: Pre-eclampsia

Numerous epidemiological studies have suggested that inadequate or unbalanced nutrition *in utero* may 'programme' the development of cardiovascular disease in adult life (Barker, 1999). In particular, low birth-weight for gestational age has been linked to the later development of hypertension, CHD and insulin resistance (Godfrey & Barker, 2000). Rat models of '*in utero* programming', such as the low-protein diet (LPD) supplemented with methionine are now well established to facilitate the study of underlying mechanisms (Desai *et al.* 1996; Langley-Evans, 1996; Rees *et al.* 1999). Recently, it has been shown that feeding this diet for the first 4 d of pregnancy led to the birth of rat pups that later developed hypertension (Kwong *et al.* 2000). These results suggest that the preimplantation embryo is particularly sensitive to diet-induced changes in maternal metabolites. The majority of studies in mammalian development have used the mouse, and as a result, there is a fuller understanding of early mouse development and mouse embryo manipulation and culture is well established (Hogan *et al.* 1994). The availability of a characterised mouse model for *in utero* programming would provide a more useful system to study the molecular basis of fetal programming.

We describe here development of such a mouse model, explicitly compared with the rat model in a test of the hypothesis that the LPD may elicit its effects through altered S amino-acid metabolism (Rees *et al.* 2000). We have compared maternal serum concentrations of methionine, threonine and homocysteine (hcy) in rats and mice, and show that the LPD induces similar changes in both species at early and late stages of pregnancy. We show that there is a marked decrease in threonine at all phases of pregnancy. We also show that there is a large increase in the concentration of hcy in the maternal serum of both species in early pregnancy and that supplementing the LPD with threonine can reduce this increase.

Experimental methods

Experimental diets

The experimental diets were as described by Langley-Evans & Jackson (1996*a*). The control diet contained

Abbreviations: hcy, homocysteine; LPD, low protein diet.

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(g/kg): casein 180, sucrose 213, cellulose fibre (Solkafloc) 50, maize starch 425, vitamin mix (AIN-76) 5, mineral mix (AIN-76) 20, maize oil 100, choline chloride 2, DL-methionine 5. The LPD contained only 90 g casein/kg with compensatory increases in sucrose (243 g/kg) and maize starch (485 g/kg). Choline chloride and DL-methionine were supplied by Sigma (Poole, Dorset, UK), all other ingredients were supplied by Special Diet Services (Witham, Essex, UK). The second experimental diet was similar in composition to the LPD described earlier but instead of 90 g casein/kg, 80 g casein was added/kg plus 10 g L-threonine (Ajinomoto Co., Inc. Japan)/kg. These diets, when fed to mice, were mixed with water, made into 1 cm^3 pellets then dried at 60°C for 48 h.

Animals

Eight virgin Rowett Hooded female rats about 6–7 weeks old were fed LPD or control diets 2 weeks prior to mating with males of the same strain. Pregnancy was confirmed by the detection of a vaginal plug and this was recorded as day 0. The animals were maintained on the experimental diets provided *ad libitum* until killed by cervical dislocation on day 4 or day 16 of pregnancy. In the day 16 group, fetuses were killed by decapitation. In all case, eight rats per group were used.

MF1 mice (3-week-old) supplied by Harlan UK Ltd (Bicester, Oxon., UK) were fed the pelleted control diet for 2 weeks before one group were transferred to LPD. This was offered for a further 2 weeks before mating with males of the same strain. Pregnancy was confirmed by the detection of a vaginal plug and this day was denoted as day 0. The mice remained on the experimental diets until being killed by cervical dislocation either on day 3 or day 17 of pregnancy. In the latter group, fetuses were weighed and killed by decapitation. In all groups, five mice were used, and food intakes were recorded with no differnces between groups being observed (results not shown). All non-pregnant animals were removed from the experiment. All experimental procedures were approved and conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986. The initiation of LPD 2 weeks prior to mating separated any stress effects of diet-change from pregnancy effects. Direct measurement of cortisol concentrations at day 3 of pregnancy confirmed that the animals were not stressed (results not shown).

Sample collection

Samples were collected from rats and mice at early and late stages of pregnancy. Following cervical dislocation, the heart was exposed and blood was collected into tubes containing 50 μl 20 mM-EDTA in PBS by heart puncture and placed on ice until centrifuged at 1000 ***g*** for 10 min at 4°C. Serum was aspirated and stored at −80°C in aliquots prior to analyses. After further dissection, the uterine horn containing either blastocyst stage embryos or fetuses was removed. Blastocysts were flushed from uterine horns on day 3 (mice) or 4 (rats) of pregnancy, isolated and counted under a Leitz dissecting microscope (Leica Microscopes, Milton Keynes, UK). After counting the total number of fetuses at later stages of pregnancy, nine were chosen at random and dissected away from their placentas. The placentas and fetuses were weighed individually. The fetuses were randomly grouped into threes and dissected so that the organs could be weighed in threes. The total weight recorded for groups of three livers, kidneys, hearts, brains and lungs were divided by three before being used in subsequent analysis.

All samples were taken in the morning to minimise any effects of circadian variation.

Amino acid analysis

Serum was thawed, vortexed and then 100 μl removed and 20 μl 1 mM-norleucine added as an external standard. Samples were analysed with a Waters Pico-Tag system (Waters Corp., Milford, MA, USA), using pre-column derivatisation with phenylisothiocyanate. Derivitives were separated on a C_{18} column using a 70 mM gradient of sodium acetate–acetonitrile buffer (pH 6·46) and detected by u.v. absorption at 254 nm and calculated using with Waters Millennium software package (Waters Corp.).

Homocysteine analysis

Total hcy was measured in 200 μl maternal serum using the DS30 hcy assay kit from Drew Scientific Ltd (Barrow-in-Furness, Cumbria, UK). Following the addition of an external standard, the disulfide bonds in the calibrant and the sample were reduced. Protein was precipitated from solution and the thiol groups in the supernatant fraction, then derivitised with a fluorescent thiol-specific dye. The fluorescent derivitives mixture was separated using the DS30 hcy analyser (Drew Scientific Ltd).

Statistical analysis

Fetal, placenta and organ weights. The individual weights for nine pups and placentas from each of five mothers per group were averaged and then the average pup weight for each mother used to calculate the mean value with its standard error for each group (n 5). Three animals in each group were not pregnant and had previously been removed from the experiment. The mean organ weight for nine pups from each of the five mothers per group was used to calculate the mean value and with its standard error (n 5). Students t test was used to assess the significance of the differences between the means.

Amino acids. Data from two independent experiments were analysed by ANOVA in Genstat 5 release 4·2 (Lawes Educational Trust, Rothamsted, Herts., UK) with diet, day of gestation, species and their interactions as treatment effects.

Homocysteine. After checking that the data were normally distributed using an Anderson-Darling normality test, the mean values with their standard errors were calculated. ANOVA was applied to the mouse and rat data separately, with diet, day of gestation and their interaction as treatment effects. Effect of diet (control, LPD or 80 g casein + 10 g threonine/kg) on the concentrations of hcy

in the maternal serum were tested by means of a one-way ANOVA. Differences between treatment means were then assessed by use of the *t* statistic calculated from the residual mean sum of squares and the residual df.

Results

Pregnancy outcome

The weights of maternal organs, placenta, fetuses and fetal organs for the mouse experiments are shown in Table 1. On day 17 of pregnancy, fetuses from the mothers fed LPD were heavier than the fetuses from the mothers fed the control diet ($P<0{\cdot}05$). There was a increase in the weight of the kidney, brain and lung ($P<0{\cdot}05$) in the LPD groups but no differences in the weight of the heart or liver. When the tissue weights were expressed as % body weight, the lung remained heavier ($P<0{\cdot}05$) in the LPD group (Table 1). The diet had no effect on numbers of preimplantation embryos isolated at day 3 (mice) or day 4 (rats) or on litter size (Table 2). There was also no relationship between the number of pups in a litter and the weight of the pups (result not shown).

Measurement of circulating amino acid concentrations

The free amino acid concentrations in mice (Table 3) and rat (Table 4) maternal serum are shown together with statistical significance for the effects for day of gestation, diet fed and species and all the associated interactions (Table 5). The concentrations of all but two amino acids (serine and glycine) differed between species. The concentrations of all but three amino acids (serine, glycine and tyrosine) altered ($P\leq0{\cdot}05$) with day of gestation. Several of the amino acids including the branched chain amino acids, together with threonine and phenylalanine, decreased with the LPD diet. There was no effect on diet × day for most amino acids except for leucine, valine and threonine. Most of the essential amino acids did not show a species × diet × day effect. Neither methionine northreonine showed a species × diet × day effect. Concentrations of methionine were higher in mice than rats.

Table 1. Maternal, placental, fetal and fetal-organ weights on day 17 of pregnancy from mice fed control or low-protein diets†‡
(Mean values with their standard errors)

Diet...	Control		LPD	
	Mean	SEM	Mean	SEM
Mothers (*n*)	5		5	
Maternal wt day 17 (g)	47·7	2·4	50·4	0·9
Fetuses per mother (*n*)	11	0·2	10	0·6
Fetal wt (g)	0·88	0·02	1·02*	0·04
Placenta wt (g)	0·14	0·01	0·15	0·01
Placenta: fetus	0·16	0·01	0·15	0·01
Organ weights (mg)				
Liver	52·5	2·9	67·3	6·3
Kidney	8·3	0·4	10·2*	0·6
Heart	5·3	0·3	6·1	0·3
Brain	51·5	1·8	59·0*	1·8
Lung	30·6	0·8	39·7***	1·6
Organ wt (% total wt)				
Liver	5·98	0·21	6·54	0·35
Kidney	0·95	0·06	1·00	0·04
Heart	0·60	0·03	0·59	0·02
Brain	5·89	0·24	5·80	0·11
Lung	3·50	0·06	3·90*	0·13

LPD, low-protein diet.
Mean values were significantly different from those of the control-diet group: *$P<0{\cdot}05$, ***$P<0{\cdot}001$.
† Control diet 180 g protein/kg, LPD 90 g protein/kg.
‡ For details of diets and procedures, see p. 472.

Homocysteine measurements

The results show a large increase in the concentrations of total serum hcy in the LPD group compared with the control group during early pregnancy in both mice (Fig. 1(A)) and rats (Fig. 1(B)). By day 17 (mice) or 16 (rats) of pregnancy the values had declined and were not different from controls. (Fig. 1(A and B). ANOVA analyses on mouse and rat results combined showed no day × diet × species interaction. Hcy concentrations were measured in mouse

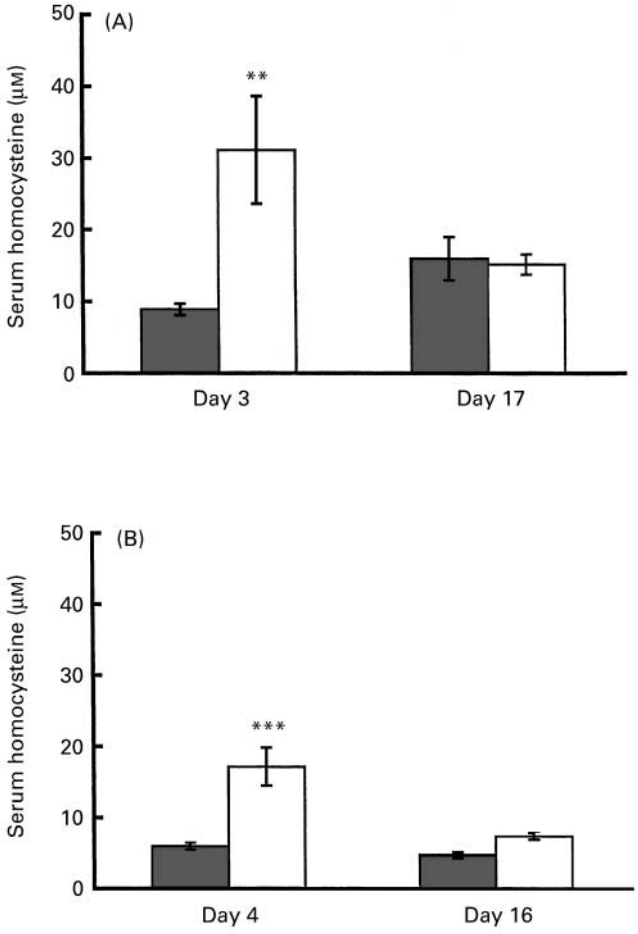

Fig. 1. Serum homocysteine in (A) mice and (B) rats, fed either control (■) or low-protein diet (LPD) (□) in early and late pregnancy. For details of diets and procedures, see p. 472. For mice: control day 3 *n* 8 and day 17 *n* 4, LPD day 3 *n* 10 and day 17 *n* 5. For rats: control day 4 *n* 7 and day 16 *n* 7, LPD day 4 *n* 7 and day 16 *n* 8. Values are means with standard errors shown by vertical bars. Mean values were significantly different from those of the control-diet group. **$P<0{\cdot}01$, ***$P<0{\cdot}001$.

Table 2. Blastocysts or fetuses (*n*) recovered per animal from control or low-protein diet-fed animals*†

(Mean values with their standard errors)

Diet...	Mouse‡				Rat§			
	Control		LPD		Control		LPD	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Blastocysts (*n*)	11	0·2	10	0·2	12	0·4	12	0·5
Fetuses (*n*)	12	0·7	11	1·6	13	1·5	13	4·0

LPD, low-protein diet.
* Control diet 180 g protein/kg, LPD 90 g protein/kg.
† For details of diets and procedures, see p. 472.
‡ Day 4 control group *n* 11, LPD group *n* 10; day 17 control and LPD groups *n* 5.
§ Day 4 control and LPD groups *n* 6; day 17 control group *n* 6, LPD group *n* 3.

maternal serum at the early stages of pregnancy in animals fed 80 g casein + 10 g threonine/kg diet and compared with the concentrations found in control and LPD animals (Fig. 2). When the mice were fed a LPD diet supplemented with 10 g threonine/kg the concentrations of hcy were similar to the concentrations of the control diet ($P > 0{\cdot}05$).

Discussion

Feeding a restricted-protein diet to rats and mice during pregnancy is known to programme disease susceptibility in offspring (Desai *et al.* 1996; Langley-Evans & Jackson, 1996*b*; Dunn *et al.* 2001). Indeed, feeding the diet described by Langley-Evans & Jackson (1996*a*) only during the preimplantation stage of pregnancy induced hypertension in rat offspring (Kwong *et al.* 2000). This result suggests that the very earliest stages of pregnancy, before the embryo has attached to the womb, may be particularly sensitive to diet-induced changes in maternal metabolites that can induce long-term programming leading to increased disease susceptibility in offspring. In order to study the molecular mechanisms involved in this phenomenon, it will be necessary to identify possible diet-induced causative agents and to systematically analyse the effects of these agents in a well characterised developmental system such as the mouse. In addition, if future mouse studies are to be meaningfully integrated with previous rat analyses, it is also important to demonstrate that the response to diet is similar in both species.

For both rat and mice, our present results showed no change in either the number of preimplantation embryos recovered at the early stages of pregnancy, or in the number of fetuses obtained at late pregnancy. This outcome suggests that the protein content of the LPD diet was sufficient to sustain normal ovulation, implantation

Table 3. Serum amino acid concentration (μM) in mice at day 3 and day 17 of pregnancy in mothers fed either control or low-protein diets*†

(Mean values with their standard errors)

Day of pregnancy...	Day 3				Day 17			
Diet...	Control		LPD		Control		LPD	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Aspartic acid	27	12	61	9	13	10	12	8
Glutamic acid	242	60	417	46	248	49	134	43
Serine	206	46	188	35	305	38	205	33
Glycine	196	33	208	25	115	27	66	23
Glutamine	642	37	880	29	788	31	776	27
Histidine	78	43	65	32	59	35	40	30
Threonine	382	54	294	41	836	44	358	38
Alanine	604	61	668	46	937	50	790	43
Arginine	157	49	137	37	68	40	81	34
Proline	288	24	120	20	212	19	205	17
Tyrosine	130	15	113	11	50	12	55	10
Valine	360	11	207	8	322	9	193	8
Methionine	349	25	321	19	414	20	275	18
Isoleucine	123	31	73	24	111	26	73	22
Leucine	192	9	116	7	146	8	97	7
Phenylalanine	82	7	62	6	61	6	51	5
Lysine	929	50	749	38	1069	41	755	35

LPD, low-protein diet.
* Control diet 180 g protein/kg, LPD 90 g protein/kg.
† For details of diets and procedures, see p. 472.

Table 4. Serum amino acid concentration (μM) in rats at day 4 and day 16 of pregnancy in mothers fed either control or low-protein diets*†
(Mean values with their standard errors)

Day of pregnancy...	Day 4				Day 16			
Diet...	Control		LPD		Control		LPD	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Aspartic acid	77	9	70	9	39	8	49	8
Glutamic acid	351	46	300	46	184	43	254	43
Serine	335	35	209	35	213	33	201	33
Glycine	277	25	203	25	183	23	200	23
Glutamine	650	29	638	29	485	27	588	27
Histidine	61	32	55	32	28	30	27	30
Threonine	790	41	437	41	551	38	471	38
Alanine	580	46	653	46	295	433	353	433
Arginine	109	37	70	37	83	34	72	34
Proline	435	18	358	18	146	17	160	17
Tyrosine	100	11	112	11	54	10	50	10
Valine	277	8	187	8	146	8	139	8
Methionine	156	19	165	19	50	18	58	18
Isoleucine	103	24	70	24	64	22	62	22
Leucine	191	7	142	7	103	7	100	7
Phenylalanine	61	6	55	6	49	5	52	5
Lysine	721	38	897	38	555	35	575	35

LPD, low-protein diet.
* Control diet 180 g protein/kg, LPD 90 g protein/kg.
† For details of diets and procedures, see p. 472.

and subsequent pregnancy. We showed that LPD in mice induces a similar increase in fetal sizes in late pregnancy (Langley-Evans & Jackson 1996*a*), with some unequal growth of internal organs as reported previously for rats (Desai *et al.* 1996). Our present results also showed that, although serum concentrations of amino acids differ between rats and mice, a similar response to diet treatments are seen with both species. For example, the marked reduction in threonine concentrations in early and late pregnancy is a common response in mice and rats (current study) and agrees with previously published results rats in (Rees *et al.* 1999; Kwong *et al.* 2000).

Recent comparative analyses of two formulations of the LPD have demonstrated that it may not be the protein composition of the diet alone that causes hypertension. Instead, it appears that it is the balance of protein and other nutrients, such as fats, carbohydrates or excessive methionine, which may determine the long-term health of offspring (Langley-Evans, 2000). Ingestion of large quantities of methionine is known to induce increased concentrations of circulating hcy (Kanani *et al.* 1999), In the LPD model, this is probably due to its production as an intermediate in the conversion of excess methionine to cysteine (Langley-Evans, 2000; Rees *et al.* 2000). Our most striking

Table 5. Statistical significance of amino acid results showing day, diet and species effects and their interactions†

	Statistical significance of effect (ANOVA)						
	Species	Diet	Day	Species × diet	Day × species	Day × diet	Species × day × diet
Aspartic acid	***	NS	***	NS	NS	NS	*
Glutamic acid	**	NS	***	NS	***	NS	*
Serine	NS	**	NS	NS	NS	NS	*
Glycine	NS	NS	NS	NS	*	NS	*
Glutamine	***	**	***	NS	***	**	***
Histidine	***	NS	***	NS	***	*	NS
Threonine	***	***	***	*	***	**	NS
Alanine	***	NS	***	***	**	**	***
Arginine	***	NS	***	NS	***	NS	NS
Proline	***	**	***	NS	**	***	NS
Tyrosine	***	NS	NS	NS	***	NS	NS
Valine	***	***	***	NS	***	***	**
Methionine	***	NS	***	**	NS	NS	NS
Isoleucine	***	**	***	**	***	NS	NS
Leucine	*	***	***	**	NS	***	NS
Phenylalanine	***	**	***	***	***	NS	*
Lysine	***	NS	***	**	***	NS	**

*$P<0{\cdot}05$, **$P<0{\cdot}01$, ***$P<0{\cdot}001$, NS $P>0{\cdot}05$.
† For details of amino acid results, see Tables 3 and 4.

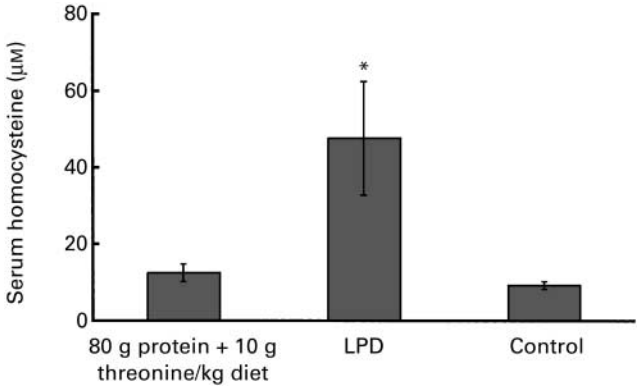

Fig. 2. Serum homocysteine in mice at day 3 of gestation fed control, low-protein diet (LPD) or diet containing 80 g protein + 10 g threonine/kg. For details of diets and procedures, see p. 472. Day 3 control *n* 6, LPD *n* 4, 80 g protein + 10 g threonine/kg *n* 3. Values are means with standard errors shown by vertical bars. Mean value was significantly different from that of the control-diet group. $^{*}P<0{\cdot}05$.

finding is the marked elevation in the concentrations of the non-protein amino acid hcy at the preimplantation stage of pregnancy in both species fed the LPD. The molecular consequences of persistent elevated concentrations of hcy have been studied in transgenic animals, engineered to have a defective tetrahydrofolate cycle. These animals show an increase in plasma hcy accompanied by high concentrations of *S*-adenosylhomocysteine, known to inhibit normal methyl transfer from *S*-adenosylmethionine. Detailed molecular analyses have shown an overall reduction in the level of DNA methylation, that closely correlates with developmental abnormalities (Chen *et al.* 2001). During the preimplantation phase of development there is extensive essential remodelling of chromatin. If preimplantation embryos are exposed to high concentrations of hcy during this time they may suffer from inhibition of methyl transferase activity and perturbations of normal genomic programming, involving DNA methylation (Dean *et al.* 2001). Deficiencies in the methyl transfer system may also alter methylation patterns of cellular proteins (Medina *et al.* 2001). This form of protein modification is important for appropriate gene expression (Ringrose & Paro, 2001). Although less well investigated, elevated concentrations of hcy may have additional deleterious effects on cellular function such as reducing rates of DNA synthesis, and endoplasmic and mitochondrial function (Medina *et al.* 2001). These effects may be equally important in defining the potential of the early embryo and could also contribute to fetal programming.

Hcy can be cleared from the circulation by re-methylation to methionine by reactions involving methyltetrahydrofolate. If there is excessive re-methylation of hcy, then pools of methylated folates become depleted, thus creating a functional folate deficiency. Clinical studies have revealed that folate deficiencies, either diet-induced or arising from polymorphisms in the genes encoding enzymes involved in the folate cycle, are associated with elevated concentrations of maternal hcy and early pregnancy loss and pre-eclampsia (Nelen *et al.* 2000). In experimental systems, maternal dietary supplements, especially designed to overcome errors in the folate cycle and methyl transfer in particular, have been shown to correct strain-dependent errors in methyl transferase activities during early development in mice and to reduce later abnormalities in the offspring (Wolff *et al.* 1998). Previous studies using the LPD have proposed a direct link with decreased concentrations of threonine and increased concentrations of hcy (Rees *et al.* 2000). Attempts to correct for LPD-induced deficiency of maternal threonine by supplementation of the LPD with threonine throughout pregnancy exacerbated the situation, however, and resulted in increased concentrations of hcy (Rees *et al.* 2000). In contrast, we find that threonine supplementation during the initial phases of pregnancy reduced maternal circulating concentrations of hcy (shown in Fig. 2). This result suggests that in animals fed the LPD, maternal concentrations of threonine may be linked to concentrations of hcy at the very earliest stages of pregnancy and that these stages may be particularly amenable to corrective dietary interventions. These findings may have important applications to clinical situations, where elevated hcy has been found to be associated with early pregnancy loss and pre-eclampsia, and suggest that pre- or peri-conceptual dietary interventions may be effective in normalising concentrations of hcy and associated adverse pregnancy outcome.

We have established that the concentrations of many of the circulating amino acids in maternal serum in rats and mice are similarly perturbed by our dietary treatment, particularly in respect to the S amino acids. We have also identified hcy as one possible agent responsible for altering 'programming' in the early embryo. An understanding of how the immediate environment of the early embryo can influence long-term developmental processes has important implications, not only with respect to maternal malnutrition, but also for all *in vitro* culture procedures associated with mammalian assisted-reproduction technologies and regenerative cloning methodologies (Aldhous, 2001). Studies of preimplantation embryos in the mouse LPD model may help to uncover the molecular mechanisms involved in long-term programming and may provide valuable insights into how to avoid the deleterious effects of toxic maternal metabolites or sub-optimal culture conditions.

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