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Maternal dietary micronutrient restriction during preconception, conception and postnatal life predispose the offspring to insulin resistance and hypertension in adult life

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ABSTRACT

To assess the effect of maternal dietary micronutrient restriction during preconception, conception and postnatal days on insulin resistance, fat metabolism and systolic blood pressure in offspring. Female weanling mice received a control or a 50% micronutrient restricted (MR) diet and mated with control males. Pups born to the dams on the restricted diet were weaned on to the restricted diet till postnatal day (PD) 360. At birth, pups from deficient dams had reduced birth weight and crown rump length. Increased fasting glucose, insulin, total cholesterol and triglycerides levels were observed in the offspring of MR group. At PD-120, MR restricted offspring had an elevated systolic blood pressure than controls. Compared with controls, total body electrical conductivity measurements indicated significantly higher body fat percentage, lower lean body mass and fat-free mass in MR offspring besides elevated plasma triacylglycerols. Maternal micronutrition restriction per se resulted in an increased body fat and in plasma triglycerides, free fatty acids and total cholesterol concentrations in the offspring. These changes seem to predispose the offspring to insulin resistance and hypertension in later life.

Key words: Micronutrient restriction, Insulin Resistance, Hypertension, TOBEC

INTRODUCTION

Many studies have demonstrated a link between fetal nutrition, low birth weight and coronary heart disease, hypertension and impaired glucose tolerance in adults[1]. The mechanism(s) by which these nutritional insults exert their effects remains unclear. To this end several animal models have been established using global caloric restriction[2] or alteration in a specific dietary component and include protein[3] fat[4] and minerals such as calcium[5] and iron (Fe)[6,7]. While protein restriction is of great significance in underdeveloped countries, and increased fat intake is a westernized problem, it is the micronutrient deficiency model that is applicable to the etiology of insulin resistance and hypertension of both developed and developing countries. Multiple vitamin deficiencies, particularly during pregnancy and/or postnatal days, are common in the developing world and maternal vitamin and mineral deficiencies are associated with low birth weights and increased rates of perinatal mortality and morbidity[8]. In view of the literature cited above, we hypothesized that maternal dietary micronutrient restriction during preconception, conception and postnatal days linked to increased oxidative stress and hyperlipidemia, body fat percentage *per se* predispose the offspring to insulin resistance and hypertension in later life.

MATERIAL AND METHODS

All animal experimental procedures were carried out with the approval from the ethical committee of the Department of Animal sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India.

Feeding and maintenance of the experimental mice

Female weanling Swiss albino mice were housed individually in polypropylene cages with wire mesh bottoms and maintained at 22 ± 2 °C, under standard lighting conditions (12-h light: dark cycle). For 12 weeks, the group of six mice was fed a basal diet (AIN-93G- composition of diet and vitamin mixture)[9], with 50% restriction of vitamin and mineral mixture (MR). The other group of 6 mice served as the control. All the animals were provided deionised water. At the end of 12 weeks of feeding, blood was collected from supra orbital sinus to determine the vitamins A, E and folic acid, and minerals such as iron, zinc, copper, magnesium, selenium and calcium in plasma; in addition to the following biochemical parameters: haemoglobin, glucose, insulin, cholesterol and triacylglycerol. After assessment of their vitamin and minerals status, the female mice were naturally mated with proven fertile control male over night and vaginal plugs were identified on the following morning and maintained on their respective diets throughout gestation.

Litter management

At term, pups born to the dams on the MR, as well as control group were weaned on the same restricted or control diet respectively. At birth, the body / placenta weight, crown rump length of neonates and gestational length of dams were recorded. In all groups, a uniform litter size of 8 pups/dam (equal number of male and females) was maintained from PD 3, until weaning on PD 21 and litters were weighed weekly from birth. Offspring born to the dams of control and MR were weaned onto the respective control and micro nutrient restricted diets. From weaning, 8 male pups from 4–5 dams of the corresponding group were maintained in each group and they consumed their respective diets and deionized water *ad libitum* until PD 360. To avoid the possible effects of estrous cycle on glucose and fat metabolism and insulin resistance, only male pups were included in this study. Daily diet intake and weekly body weights were recorded in mothers and offspring.

Measurements in plasma

Blood collected from offspring on PD 30, 60, 90, 180 and 360 were utilized for the measurements of biochemical parameters such as Vitamins A, E, folic acids, iron, calcium were measured in maternal plasma by using commercially available kits from Sigma, St. Louis, USA. Zinc, copper, magnesium concentrations were determined using flame atomic absorption spectrophotometer (Perkin-Elmer model 460, Norwalk, CT), as described previously[10,11]. Selenium concentrations were determined by flameless atomic absorption spectroscopy after a sampling dilution procedure, with a Perkin-Elmer model 5100 fitted with a HGA 600 graphite furnace[12]. Plasma glucose and high density lipoprotein (HDL) cholesterol, free fatty acids, serum triglycerides (TG), total cholesterol (TC) levels were measured by using the using commercially available kits from Ranbaxy, India. LDL-cholesterol and VLDL cholesterol were calculated by using Friedewald formula[13]. Plasma insulin was measured using a radioimmunoassay kit from BRIT, Mumbai, India.

Oral Glucose Tolerance Test (OGTT)

An oral glucose-tolerance test (OGTT) was performed in the offspring on PD 90, 180 and 360. After an overnight fast, glucose (300 gm/l) was administered orogastrically at a dose of 2.5 g/kg body weight and blood samples were obtained from the orbital sinus at 0, 60 and 120 min for determination of plasma glucose and insulin concentrations.

Oxidative stress and antioxidant status

On PD 360, the animals were killed by decapitation. The livers were dissected out immediately, washed thoroughly with ice-cold 0.9% NaCl, frozen in liquid nitrogen and stored at -80 °C until the analysis. About 1 g liver (from the biggest lobe) was weighed out, minced and homogenized (10% w/v) in 50 mmol/l phosphate buffer (pH 7.0). The homogenate was centrifuged at 1000 g for 20 min at 4 °C. A part of the supernatant was used to estimate lipid peroxidation and protein carbonyls. The remaining supernatant was further centrifuged

at 12,000 g for 20 min at 4° C to obtain the post mitochondrial supernatant. This was used to estimate reduced glutathione (GSH) and activities of the following antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx).

Fasting blood samples were collected on PD 360 from all the MR and control group animals, for the measurement of lipid peroxide and total glutathione. Lipid peroxidation was measured by the thiobarbituric acid color reaction for malondialdehyde (MDA)[14]. Protein carbonyl content was measured spectrophotometrically using 2,4-dinitro-phenyl-hydrazine[15]. Total GSH (GSH + GSSG) was determined enzymatically in the acidic protein-free supernatant[16]. The assay of oxidized glutathione (GSSG) was performed after masking–reduced glutathione (GSH) by adding 2-vinylpyridine to the deproteinized extract. After this procedure, GSSG was also enzymatically determined. Catalase was assayed by monitoring the disappearance of H_2O_2 at 240 nm[17]. A coupled enzyme procedure, with cumene H_2O_2 as a substrate, was used to determine total GSH-Px activity[18]. The protein content was determined using the modified Lowry method[19].

Blood pressure measurements

Systolic blood pressure was determined at 120, 180, 260 and 360 days of age in both control and MR group by tail cuff plethysmography using an IITC model-229 blood pressure monitor (Linton Instruments, UK). After 1-hour acclimatization, recordings were made "blind" (mean of four per mice) by coding animals and if heart rate exceeded 480 beats/minute (indicative of stress), results were discarded.

Body composition of the offspring

Total body composition of the offspring was determined at PD 180 and 360 using a Total Body Electrical Conductivity (TOBEC) - small animal body composition analysis system (EM-SCAN, Model SA-3000 multi detector, Springfield, IL, USA)[20].

Statistical analysis

Data are expressed as means ± SE. Statistical analyses were performed using paired Students't-test or one way ANOVA followed by Bonferroni analysis where ever appropriate. Statistical significance was determined at the 0.05 level.

RESULTS

Parameters in mothers

Maternal growth, mineral, vitamin status, lipid profile and glucose homeostasis.

At the end of 12 weeks of feeding there was no significant difference in the daily food intake and body weight gain between the control and MR groups. However, hemoglobin concentration was significantly decreased ($P < 0.001$) in MR animals than in control. In addition, food intake was not significantly different between the MR and control group throughout growth, pregnancy and lactation. There was, however, a significant decrease in the plasma concentration of copper ($P<0.001$), zinc ($P<0.001$), iron ($P<0.001$), magnesium ($P<0.001$), calcium ($P<0.01$), Selenium ($P<0.001$), vitamin-E ($P<0.001$), vitamin-A ($P<0.05$) and folic acid

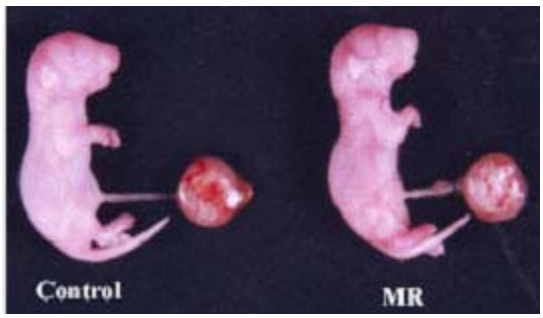


Fig. 1a: Differences in size of the neonate of Control and MR along with placenta

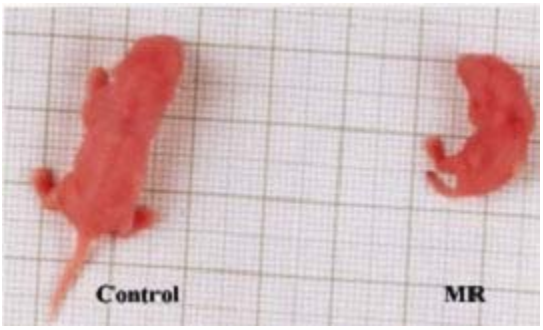


Fig. 1b: Differences in crown rump length of offspring of Control and MR

(P<0.001) in MR animals compared with controls after 12 weeks of feeding (Table 1). There were no significant differences between the concentrations of fasting glucose and HOMA index in control and MR group. However there was a slight increase in fasting plasma insulin level in MR group as compared with control. There were no statistical differences in plasma total cholesterol levels; however there was a significant increase in the plasma triglyceride levels in MR mice compared with control.

Table 1: Diet intake, physical and Biochemical parameters in mother at the end of 12 weeks

Parameter	Control	MR
Vitamin E (µmol/l)	55 ± 2.9	35 ±2.3***
Folic acid (mmol/l)	453 ± 12	403 ± 16***
Vitamin A (µmol/l)	1.6 ± 0.08	1.0 ± 0.11*
Copper (µg/ml)	1.53 ± 0.07	0.9 ± 0.04***
Zinc (µg/ml)	1.64 ± 0.05	0.78 ± 0.05***
Iron (mg/ml)	4.59 ± 0.12	3.1 ± 0.09***
Selenium (mmol/l)	4.0 ± 0.17	3.0 ± 0.29***
Magnesium (mg/ml)	26 ± 0.98	17 ± 0.85***
Calcium (mg/ml)	190 ± 11	160 ± 14**
Hemoglobin (g/dl)	13.9 ± 0.21	10.1 ± 0.02***

Values represent mean ± s.d, of 8 mice per group. *** p<0.001, ** p<0.01, * p<0.05 vs. control; by student's t test.

Table 2: Placental weight, mean birth weight and crown rump length of offspring in MR and control group

Parameter	Control	MR
Placenta weight (mg)	120 ± 2.9	88 ± 3.4***
Neonates birth weight (g)	2.21± 0.06	1.65 ± 0.11***
Neonates crown rump length (cm)	3.24 ± 0.01	2.61 ± 0.10**

Values represent mean ± s.d, of 8 mice per group. *** p<0.001, ** p<0.01, * p<0.05 vs. control; by student's t test.

Table 3: Levels of fasting glucose and fasting insulin in offspring of MR and control groups on PD 180 and 360

Parameters	PD 180		PD360	
	Control	MR	Control	MR
Fasting Glucose (mmol/l)	4.2 ± 0.12	4.6 ± 0.08	4.6 ± 0.27	4.6 ± 0.17
Fasting Insulin (pmol/l)	590 ± 60	770±70***	620 ± 60	730 ± 10***

Values represent mean ± s.d, of 8 mice per group. *** p<0.001, ** p<0.01, * p<0.05 vs. control; by student's t test.

Maternal dietary micronutrient restriction impairs fetal growth, litter size and placental weight

Feeding of restricted micronutrient during preconception and conception periods resulted in fetal intrauterine growth retardation (Fig. 1A and 1B). Mean birth weight (P <0.001) and crown rump length (P <0.001) were significantly lower in the MR group than in control. Placental weight was decreased by 26 % (P <0.001) in MR group as compared with control (Table-2). However there was no difference in gestational length between two groups.

Parameters in offspring

Growth characteristics of the offspring

The offspring of the MR dams which had a lower birth weight (P<0.05) continued to weigh less until PD 360 (Fig. 2).

Micronutrient status in offspring

Chronic micronutrient dietary restriction continuation during postnatal days significantly decreased the plasma concentration of Cu, Zn, Fe, Mg, Se, Ca, folic acid and Vit E, A in MR group on PD 30, 60, 90, 180 and 360, (p<0.001) obviously

Table 4: Results of Glucose tolerance test showing the levels of Plasma glucose and plasma insulin in offspring of MR and control groups on PD 180

Parameters	Control			MR		
	0 Min	60 Min	120 Min	0 Min	60 Min	120 Min
Plasma Glucose (mg/dl)	84 ± 4	120 ± 7	92 ± 8	97 ± 5***	129 ± 6***	138 ± 5***
Plasma Insulin(ng/ml)	1.3 ± 0.07	1.4 ± 0.04	1.4 ± 0.07	1.7 ± 0.04***	1.9 ± 0.02***	2.3 ± 0.04***

Values represent mean ± s.d, of 8 mice per group. *** p<0.001, ** p<0.01, * p<0.05 vs. control; by student's t test.

Table 5: Results of Glucose tolerance test showing the levels of Plasma glucose and plasma insulin in offspring of MR and control groups on PD 360

Parameters	Control			MR		
	0 Min	60 Min	120 Min	0 Min	60 Min	120 Min
Plasma Glucose (mg/dl)	90 ± 5	118 ± 4	87 ± 6	129 ± 6***	161 ± 7***	175 ± 3***
Plasma Insulin(ng/ml)	1.6 ± 0.07	1.7 ± 0.07	1.7 ± 0.09	3.1 ± 0.05***	3.7 ± 0.08***	4.1 ± 0.04***

Values represent mean ± s.d, of 8 mice per group. *** p<0.001, ** p<0.01, * p<0.05 vs. control; by student's t test.

Table 6: Lipid Profile of the offspring in MR and Control on PD 180 and 360

Parameters	PD 180		PD360	
	Control	MR	Control	MR
Total Cholesterol (mmol/l)	1.67 ± 0.15	2.06 ± 0.03***	1.86 ± 0.16	2.60 ± 0.28***
HDL Cholesterol (mmol/l)	1.48 ± 0.11	1.17 ± 0.04***	1.26 ±0.13	0.92 ± 0.09***
Triglycerides (mmol/l)	0.51 ± 0.09	0.67 ± 0.06***	0.59 ± 0.05	0.72 ± 0.08***

Values represent mean ± s.d, of 8 mice per group. *** p<0.001, ** p<0.01, * p<0.05 vs. control; by student's t test.

indicating the sub normal supplementation through the diet .

Glucose Homeostasis

There were no significant differences among control and MR groups in fasting plasma glucose and insulin levels till PD 90. On PD 180 and 360, the MR group had significantly higher fasting glucose ($p<0.01$) and insulin levels than controls (Table 3). Similarly, oral GTT results also indicated significant changes on PD180 ($p<0.001$) and 360 ($p<0.001$) confirming the onset of Insulin resistance in later life (Table 4, 5).

Lipid Profile and oxidative stress

Levels of total cholesterol, HDL and triglycerides were significantly higher on PD 180 and 360 ($p<0.001$) in the MR group than the control indicating impairment in the lipid metabolism (Table 6). Chronic maternal micronutrient restriction resulted in a significant increase in lipid peroxides (TBARS) and a decrease in GSH ($P< 0.001$) levels in both plasma and liver. Compared with the control group, blood GSSG ($P< 0.001$) and GSSG/GSH ratio ($P< 0.001$) were also significantly higher in the MR group. Protein carbonyl levels in the liver was also significantly elevated than in control group.

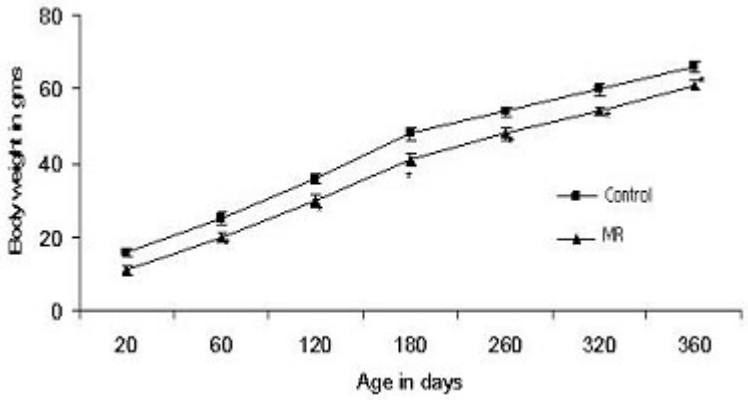
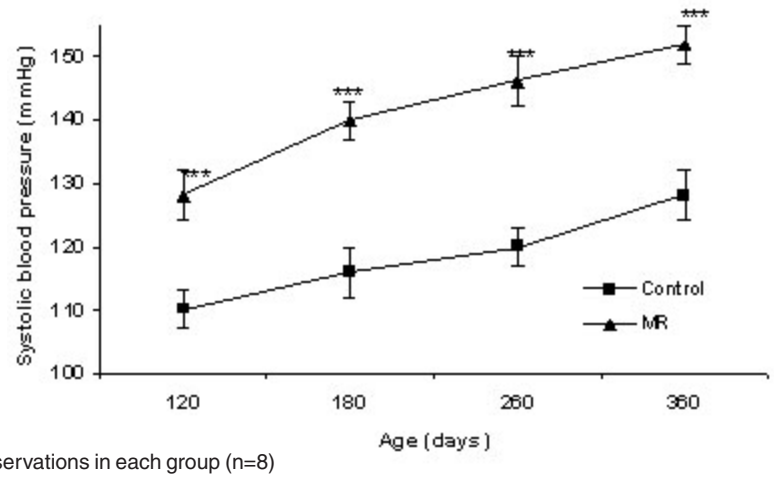


Fig. 2: Body weights of the offspring at weaning and on post-natal days 20, 60, 120, 180, 260 and 320. Results are presented as means ± s.d; * $p<0.05$ vs. control



[1] Number of observations in each group (n=8)

[2]Control· control diet throughout; MR, mineral and vitamin restriction diet throughout

Fig. 3: Systolic blood pressure in offspring of MR and control group mice on PD 20, 180, 260 and 360. Results are presented as means ± s.d; *** $p<0.001$ vs. controls[1-2]

Hepatic and plasma SOD, Catalase and GPx activities were significantly higher in micronutrient restricted offspring than in control (P<0.001) (Table 7 and 8).

Body composition

Compared with control group, body fat percent (p<0.001) and adiposity index was significantly higher (p<0.001) in offspring of MR

Table 7: Indices of oxidative stress, antioxidants and antioxidant enzyme levels in the liver of offspring of MR and control at PD 180 and 360

Parameters	PD 180		PD360	
	Control	MR	Control	MR
TBARS (mmol/mg of tissue)	1.3 ± 0.28	2.4 ± 0.07***	1.5 ± 0.16	2.8 ± 0.08***
Protein carbonyls (nmol/mg protein)	5.32 ± 0.82	6.92 ± 0.24***	5.95 ± 0.15	7.14 ± 0.25***
Cu-Zn -SOD (units/mg of protein)	16.1 ± 0.74	20.1 ± 0.23***	17.8 ± 0.93	21.7 ± 0.52***
Catalase (units/mg of protein)	36 ± 2.7	50 ± 4.8***	40 ± 4.3	56 ± 3.3**
GSH-Px (units/mg of protein)	15 ± 0.3	24± 0.1***	18 ± 0.9	27 ± 0.8***
GSH (µmol/mg of protein)	692 ± 7.2	520 ± 10***	719 ± 6.2	540 ± 9.2***

Values represent mean ± s.d, of 8 mice per group. *** p<0.001, ** p<0.01, * p<0.05 vs. control; by student's t test.

Table 8: Indices of oxidative stress, anti oxidant and antioxidant enzyme levels in the hemolysate of offspring at PD 180 and 360

Parameters	PD 180		PD360	
	Control	MR	Control	MR
TTBARS, µmol/L	2.0 ± 0.15	3.1 ± 0.17***	2.25 ± 0.28	3.93 ± 0.19***
GSH-Px, U/L	5700 ± 120	6700 ± 117***	6200 ± 150	6910 ± 196***
GSSG, µmol/L	4.72 ± 2.3	7.23 ± 2.8***	5.25 ± 1.9	9.18 ± 4.5***
GSH, µmol/L	679.2 ± 23.4	552 ± 62***	851.2 ± 46.1	740.5 ± 50.5***
GSSG/GSH, X 10^3	5.8 ± 0.3	9.1 ± 0.2***	6.2 ± 0.2	10.2 ±0.3***
Catalase, KU/g L	67.8 ± 11	93 ± 4.8***	80.8± 10.1	110± 6.2***

Values represent mean ± s.d, of 8 mice per group. *** p<0.001, ** p<0.01, * p<0.05 vs. control; by student's t test.

Table 9: Adiposity Index - body fat percentage, lean body mass (LBM) and Fat free mass (FFM) in the offspring of Control and MR at PD 180 and 360

Parameters	PD 180		PD360	
	Control	MR	Control	MR
Body Fat %	9 ± 0.5	13.2 ± 1.0***	13.5 ± 0.5	17.3 ± 1***
Lean Body Mass (g)	27 ± 1	20 ± 0.8***	31 ± 2	23.8 ± 1***
Fat Free Mass (g)	16 ± 0.8	11 ± 0.5***	18.6 ± 0.9	12.5 ± 0.5***
Adiposity Index	4.77 ± 0.288	6.26 ± 0.166***	4.22 ± 0.72	5.36 ± 0.57 ***

Values represent mean ± s.d, of 8 mice per group. *** p<0.001, ** p<0.01, * p<0.05 vs. control; by student's t test.

group on PD 180 and 360. Other markers of adiposity like lean body mass and fat-free mass were significantly lower (P<0.001) on PD 180 and 360 (Table-9).

Hypertension

Systolic blood pressure in offspring of MR group was significantly elevated (P<0.001) on PD 120, 180, 260 and 360 than the control indicating hypertension (Fig. 3).

DISCUSSION

The effect of micronutrient restriction during preconception, conception and postnatal days appears to predispose the offspring to insulin resistance and hypertension in later life. Chronic maternal dietary micronutrient restriction affected the birth and placental weights of the offspring. Interestingly, decrease in the bodyweight was continued at weaning and thereafter, in micronutrient restricted group. Our data support the few previous experimental[21,22] and epidemiological[23] studies that showed, prenatal under nutrition can affect body weight in adulthood. In our experiment, maternal micronutrition restriction reduced the placental weight, which might be a causative factor responsible for the reduced birth weight in micronutrient restricted group by modulating placental nutrient transfer and metabolism. Chronic maternal micronutrient restriction and its postnatal continuation in the offspring had no discernable effect on their fasting plasma glucose and insulin levels or oral glucose tolerance until PD 180. However, elevated fasting glucose, insulin and impaired GTT were observed from PD-180 onwards. These observations are comparable with the previous reports on maternal protein and calorie malnutrition[24], which showed that maternal protein or caloric restriction affected glucose tolerance in the offspring in experimental rat model. Beside insulin resistance, maternal micronutrient restriction increased the systolic blood pressure from PD120. These observations are in agreement with Woodall and coworkers[25], who showed that maternal undernutrition or iron restriction in rats elevated blood pressure in adult life.

Micronutrient restriction moderately increases the circulating total, LDL, VLDL cholesterol and triglycerides levels. The increased triglyceride levels in MR could also be linked to the high formation of glycerol-3-phosphate leading to an increased synthesis of VLDL by liver. However, HDL cholesterol levels were moderately decreased in MR group. This finding support the hypothesis, based on epidemiological studies in humans, that restricted growth before birth can have long-term deleterious effects on cholesterol homeostasis[26].

The finding in our study that both Lean Body Mass (which includes tissue-associated fat) and Fat Free Mass showed a significant decrease in MR compared with control appears to suggest that tissue-associated fat may be decreased in MR mice, whereas visible fat percent increased. These changes in body fat % are in agreement with those found by Lucas *et al.*[27]. The high body fat% along with low body weight in the MR offspring observed here appear to be similar to those reported in the "thin-fat" neonatal phenotype[28] seen in developing countries such as India, an abnormal condition attributed to maternal malnutrition[29].

There are reports that lipid abnormalities and insulin resistance are associated with an abnormal antioxidant defense and increased oxidative stress[30]. It has also been suggested that oxidative stress plays a role in the development of insulin resistance[31]. In line with these reports, we observed an increase in oxidative stress and decreased antioxidant status in micronutrient restricted mice compared with control.

Zinc is a biological antioxidant[32], and its depletion can lead to oxidative stress[33] and reduced insulin sensitivity[34]. In particular, this metal is associated with the apoprotein of superoxide dismutase; thus its depletion could alter the protein as previously shown[35]. Thus, micronutrient restricted mice had increased oxidative stress. The increased catalase activity in MR is an indicator of increased production of peroxide radicals. This probably corroborates the importance of non-enzymatic antioxidants (especially vitamins and trace elements) as the primary line of defense against oxidative stress suggesting that maternal micronutrient restriction modulates/programs antioxidant enzymes in the offspring to cope with the increased oxidative stress[36]. It appears that the increased oxidative

stress observed in maternal and/or post-natal micronutrient restriction could be involved in the changes in adiposity and/or lipid metabolism, the well-recognized forerunners of insulin resistance and hypertension.

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