

DOES THE MATERNAL MICRONUTRIENT DEFICIENCY (COPPER OR ZINC OR VITAMIN E) MODULATE THE EXPRESSION OF PLACENTAL 11  $\beta$  HYDROXYSTEROID DEHYDROGENASE-2 *PER SE* PREDISPOSE OFFSPRING TO INSULIN RESISTANCE AND HYPERTENSION IN LATER LIFE ?

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**Abstract :** The relevance of maternal macronutrient deficiency on developmental origin of health and adult disorders has been well studied but not that of micronutrients. We hypothesized that chronic maternal dietary mineral (copper or zinc) or vitamin E restriction modulates the expression of placental 11  $\beta$  hydroxysteroid dehydrogenase-2 (11  $\beta$  HSD-2) *per se* predisposing the offspring to insulin resistance (IR) and hypertension in later life. Female weaning Swiss albino mice received a control or a 50% of Vitamin-E or Zn or Cu restricted diet and mated with control males. Pups born to the dams on the restricted diet had significantly ( $P < 0.001$ ) reduced body weight and crown rump length. These offsprings were weaned on to the restricted diet till postnatal day 180. Glucose intolerance in association with hyperinsulinemia (IR), hyperlipidemia and increased systolic blood pressure were recorded in all the offsprings of micronutrient restricted groups. Placental 11  $\beta$  HSD-2 expression was attenuated, while activities of glucocorticoid -insensitive enzymes were unchanged in all the restricted groups. Thus, the present study reiterates the importance of micronutrients during pregnancy because chronic maternal micronutrient deficiency may alter placental 11  $\beta$  HSD-2 expression and predispose the offspring to IR and hypertension in later life.

**Key words :** insulin resistance    copper restriction    vitamin E restriction  
                                 fetal malnutrition    hypertension                                    zinc restriction

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## INTRODUCTION

Correlation between low birth weight in humans and susceptibility to a number of adult chronic disease, including coronary heart disease, stroke, high systolic blood pressure and non-insulin dependent diabetes mellitus, has been identified (1–2). Size at birth is thought to be linked to chronic disease in adulthood via “programming”. According to Lucas (3), “programming” occurs because of an early stimulus or insult, operating at a critical or sensitive period of development resulting in a permanent or long-term change in the structure or function of the organism. Recent epidemiological observations from several populations around the world, have indicated that adult risk of hypertension and coronary heart disease is strongly linked to intrauterine factors (4). Intrauterine growth is primarily determined by parental genetic factors and maternal size, metabolic, endocrine and nutritional status. Of these factors, nutrition has the most variable influence. Maternal undernutrition is associated with retarded fetal growth and reduced birth weight of new born (5–6).

Animal studies broadly confirm the epidemiological findings and support the hypothesis that maternal undernutrition in pregnancy “programmes” later hypertension and Type II diabetes (7). Restriction of maternal food intake to 30% of *ad libitum* through out the pregnancy (8) or feeding low protein diet during preimplantation period or glucocorticoid excess during pregnancy in rats results in the birth of growth-retarded offsprings (9), which have raised blood pressure in adult life. Thus the role of maternal macronutrient malnutrition but not

of micronutrients has been well documented in the fetal origin of adult disease. We hypothesize that chronic maternal Zn, Cu and Vitamin E restriction modulate the expression of placental 11  $\beta$  hydroxysteroid dehydrogenase-2 and *per se* predispose the offspring to insulin resistance and hypertension in later life.

## MATERIAL AND METHODS

### Animals

All procedures involving animals were carried out under the Institute animal ethics committee approval. Animals were handled using the laboratory animal welfare guidelines (10). The experimental study was carried out using weaning female Swiss albino mice weighing between 10–12 grams. The animals were housed individually and maintained at 22°C on a 12 h light and dark cycle. The acclimatized weaning female mice were divided into four groups of fourteen animals each, with equal mean body weights.

### Experimental treatments

Group I (control) animals were fed on complete diet. In Group-II, III and IV animals, were fed copper (Cu-R), zinc (Zn-R) and vitamin E (Vitamin-E R) restricted diet, respectively (50% restriction as compared to complete diet). The mice were fed on modified American Institute of Nutrition-93 G basal diets (11), containing casein as the source of protein and corn starch and lactose as the source of carbohydrates, for the entire experimental period. Each group of animals was fed *ad libitum* from weaning the respective restriction diet schedule and received deionized water.

After 12 weeks of feeding respective diets, blood was collected from supra orbital sinus, to determine the concentrations of hemoglobin, copper, zinc, vitamin-E, glucose, insulin, cholesterol, and triglycerides using commercially available kits from Sigma Chemical Co., St. Louis, MO. After 12 weeks of feeding respective diets, the female mice were naturally mated with proven fertile control male over night and vaginal plugs were examined the following morning. Plug positive females were then transferred to single cages and fed *ad libitum* respective restricted diets (Groups II to IV) or control diet (Group I). Pregnant mice were weighed at four days interval and daily food consumption of each animal was recorded throughout the entire gestational period.

#### Litter management

At term pups born to the dams on the restricted diet as well as control group were weaned on the same restricted or control diet respectively. At birth, the body weight, crown rump length of neonates and gestational length of dams were recorded. Within 12 hr of birth, offspring sex was determined by examination of external genital morphology. In all groups, a uniform litter size of 8 pups/dam (equal number of male and females) was maintained from postnatal d 3, until weaning on postnatal d 21 and litters were weighed weekly from birth. 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 postnatal day 180. To avoid the possible effects of estrous cycle on glucose and fat metabolism and IR, only male pups were included in this study.

#### Biochemical measurements in plasma of offspring on postnatal day 180

After overnight food deprivation, blood was collected from the supraorbital sinus of pups on postnatal day 180; plasma was separated and stored at  $-20^{\circ}\text{C}$  until further analysis. Glucose (glucose oxidase/ peroxidase kit), triglycerides (glycerol phosphate oxidase kit), total cholesterol (cholesterol oxidase-peroxidase kit) were measured in plasma using enzymatic assay kits. Plasma insulin was measured by RIA using a kit from BRIT, India.

#### Glucose tolerance test

An i.p. glucose tolerance test (IPGTT) was performed on 8 pups from each group on postnatal day 180. Briefly, after overnight food deprivation (16 h), glucose (250 g/L) was administered i.p. as a bolus, at a dose of 1 g/kg body weight (12), and blood samples were collected for determining plasma glucose (0, 30, 60, 90 and 120 min) and insulin (0, 60, 120 min) concentrations.

#### Physiological index of insulin resistance

The homeostasis model assessment for insulin resistance (HOMA-IR) index was calculated based on the values of fasting glucose and insulin concentrations, using the following formula.

$$\text{HOMA-IR} = \left\{ \frac{\text{Fasting insulin } (\mu\text{U/ml}) \times \text{fasting glucose (mmol/l)}}{22.5} \right\}$$

#### Biochemical measurements in placentas

In 50% of animals, pregnancies (n=7/each diet group) were terminated on day 19 of

gestation. Placenta and fetuses were excised and weighed separately.

#### Western Blot analysis of 11 $\beta$ HSD-2

Placental samples (approximately 100 mg) were homogenized using a Polytron Tissue homogenizer (PT 10 probe; position 5, 15 s) in 1 ml ice cold extraction buffer A: 20 mmol/l Tris, 137 mmol/l NaCl, 2.7 mmol/l KCl, 1 mmol/l CaCl<sub>2</sub>, 10% Glycerol, 1% Igepal, 45 mmol/l sodium orthovanadate (Na<sub>3</sub>VO<sub>4</sub>), 0.2 mmol/l phenylmethylsulphonyl fluoride, 10  $\mu$ g/ml leupeptin, 1.5 mg/ml benzamide, 50  $\mu$ g/ml aprotinin, 50  $\mu$ g/ml pepstatin A (in DMSO), pH 8.0. Homogenates were placed on ice for 20 min, centrifuged in an Eppendorf centrifuge (12000 r.p.m for 20 min, at 4°C) and the supernatants stored at -20°C until required for analysis. Samples (20–30  $\mu$ g of total protein) were subjected to SDS-PAGE using a 12% for resolving gel, with 6% stacking gel. After SDS-electrophoresis, resolved proteins were transferred electrophoretically to nitrocellulose membrane and then blocked with Tris buffered saline supplemented with 0.05% Tween (TBST) and 5% (w/v) non fat powdered milk for 2 hr at room temperature. The nitrocellulose blots were incubated over night at 4°C with monoclonal antisera raised 11  $\beta$  HSD-2 (1:2000 dilution), washed with 0.05% Tween in TBS (3 X 5 min) and incubated with the alkaline phosphatase-linked secondary antibody IgG anti-rabbit (1:2000), in 1% (w/v) non-fat milk in TBST for 1 or 2 h respectively at room temperature. Bound antibody was visualized using substrate. The signals quantified by scanning densitometry and analyzed with molecular analyst software.

#### Activities of 11 $\beta$ HSD, Glutamine synthase, Aryl hydrocarbon hydroxylase, Malate dehydrogenase and Pyruvate kinase in placenta

Placentas were homogenized in Krebs-Ringer Buffer (K.RB), pH 7.4. This homogenate was used for the estimation of the activities of 11  $\beta$  hydroxy steroid dehydrogenase (13), corticosterone inducible glutamine synthase (GSase), steroid insensitive aryl hydrocarbon hydroxylase (AHH), malate dehydrogenase (MD ase) and pyruvate (PK ase) kinase (14–15).

#### Blood pressure measurements

Systolic blood pressure was determined at postnatal day 180 in both control and restricted 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/minutes (indicative of stress), results were discarded (16).

#### Statistical analysis

All values are presented as mean $\pm$ s.d. Comparisons between two independent sets of data was performed with unpaired Student's t test. The criterion for significance was set at P<0.05.

## RESULTS

#### Parameters in mother

##### *Maternal growth, mineral and vitamin status, lipid profile and glucose homeostasis*

At the end of 3 months, there was no

significant difference in the average daily food intake and body weight between the control and the micronutrient restricted groups. The average food intake was not significantly different, although slightly less in the restricted groups. A significant difference in the concentration of Cu, Zn, Vitamin-E, glucose, insulin and HOMA-IR levels in respective restricted groups was observed. However the maternal total cholesterol, hemoglobin and triacylglycerols levels between the restricted and control groups (Table I) were unchanged.

*Reproductive performance of dams*

Conception was 100% in both control and

the micronutrient restricted groups. The abortion rate was 15%, 20% and 45% respectively in Cu-R, Zn-R and Vitamin-E R animals. The litter size was significantly different between the groups (Table-II). The rate of still births (3–4%) and death during lactation (4–5%) was not significant between groups compared with none in control. However there was no significant change observed in gestation days of restricted and control groups (Table-II).

*Maternal body weight and food intake during gestation*

The food consumption and maternal weight gain did not significantly change till day 10 of pregnancy in micronutrient

TABLE I: Diet intake, physical and biochemical parameters in female mice fed on control and micronutrient restricted diet for 12 weeks from weaning.

Parameter	Control	Cu-R	Zn-R	Vitamin-E R
Food intake (g/day)	8.0±1.5	7.8±2.1	7.5±1.9	7.7±1.4
Body weight (g)	30±2.2	27±2.6	29±1.7	29±2.7
Vitamin E (µmol/l)	55±2.9	53±2.3	54±3.1	33±3.3***
Copper (µg/ml)	1.53±0.07	0.9±0.04***	1.41±0.06	1.44±0.05
Zinc (µg/ml)	1.64±0.05	1.58±0.05	1.01±0.04***	1.55±0.07
Fasting glucose (mg/dl)	85±3.4	110±3.7***	108±4.1***	102±3.2**
Fasting insulin (ng/ml)	1.1±0.06	1.7±0.04**	1.8±0.06**	1.6±0.07**
HOMA-IR	11.8±0.9	19.5±0.7***	19.7±0.6***	17.5±0.9***
Total cholesterol (mg/dl)	185±3.5	192±3.9	190±3.5	190±4.9
Triglycerides (mg/dl)	110±3.3	116±4.1	115±4.3	116±3.9
Hemoglobin (mmol/l)	7.8±0.2	7.6±0.1	7.7±0.2	7.6±0.1

Values represent mean±s.d., from n=14/each group; \*\*P<0.01, \*\*\*P<0.001 vs control; Student's t test. HOMA-IR - Homeostasis model assessment for insulin resistance.

TABLE II: Body weight, crown rump length, litter size of offspring of dams fed on control or weaned on three different dietary micronutrient restriction groups.

Parameter	Control	Cu-R	Zn-R	Vitamin-E R
Body weight (g) (n=20)	2.21±0.06	1.45±0.11***	1.58±0.17***	1.56±0.27***
Crown rump length (cm) (n=20)	3.24±0.01	2.51±0.10***	2.64±0.14***	2.49±0.32***
Litter size (n=7)	13.8±0.41	8.10±2.60***	7.50±2.98***	7.5±2.76***
Gestational period (day) (n=7)	20±0.02	21.5±0.93	21±0.96	21±0.61

Values represent mean±s.d., n, number of observations for each group. \*\*P<0.001 vs control; by Student's t test.

restricted groups; but these two parameters significantly decreased ( $P < 0.001$ ) in all the restricted mice during late gestation period (Fig. 1A and B). The maternal weight gain pattern was similar in all the four micronutrient restricted groups and controls.

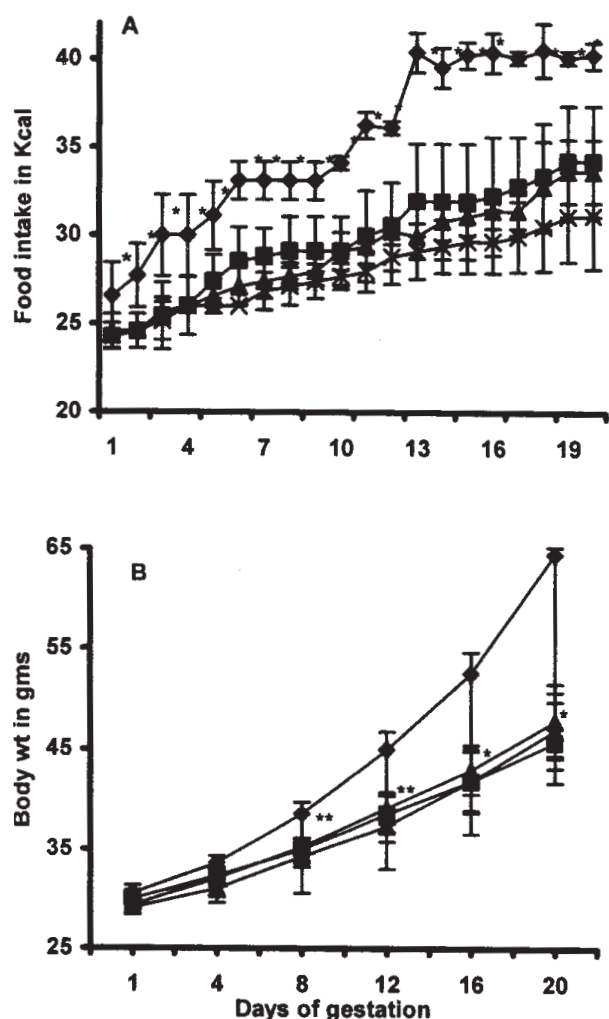


Fig. 1: Food intake (A) and Body weights (B) in control ( $\square$ ) and micronutrient restricted ( $\square$  Cu-R;  $\square$  Zn-R;  $\times$  Vitamin-E-R) mice. Results are presented as mean  $\pm$  s.d., from n=7; \*P < 0.05; \*\*P < 0.01 vs control by Student's t test.

#### Parameters in offsprings

##### Fetal growth and placental weight

In the present study Cu or Zn or Vitamin-E restricted diet during preconception and conception period lead to fetal intrauterine growth retardation. This observation is in conformity with similar studies earlier. The birth weight and crown rump length of pups were significantly lower in all the micronutrient restricted groups (Table-II), but severe in Cu-R. Placental weight and protein concentrations at day 19 of gestation were considerably lower in all restricted groups (Fig. 2A and B).

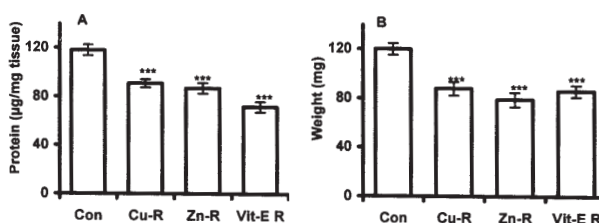


Fig. 2: Placental weight (A) and protein concentration (B) in control ( $\square$ ) and micronutrient restricted ( $\square$  Cu-R;  $\square$  Zn-R;  $\times$  Vitamin-E-R) mice. Results are presented as mean  $\pm$  s.d., from n=7; \*\*\*P < 0.001 vs control by Student's t test.

##### Placental 11 $\beta$ hydroxysteroid dehydrogenase-2 expression

On day 19 of gestation, levels of 11  $\beta$  HSD-2 protein expression in placentas were markedly lower by 32% in Cu-R; 40% in Zn-R and 41% in Vitamin-E R ( $P < 0.001$ ) group as compared with the control (Fig. 3).

##### Activities of 11 $\beta$ HSD, GSase, AHH, MDase and PKase in placenta

Placental activity of 11  $\beta$  HSD was

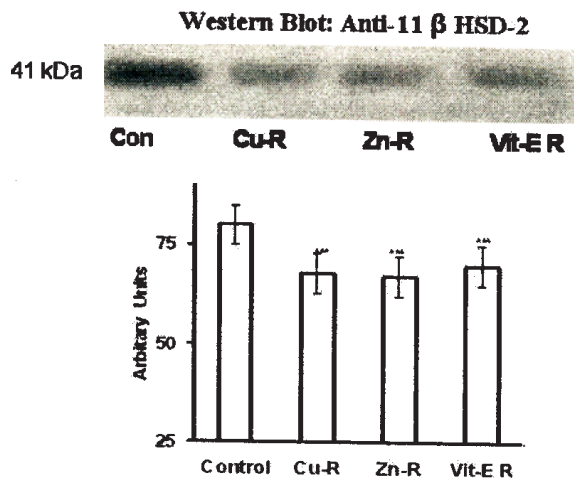


Fig. 3 : 11 β hydroxysteroid dehydrogenase-2 protein expressions in placentas of 19-day-old control, Cu-R, Zn-R, Vitamin-E-R mice. Results are presented as mean±s.d., from n=7; \*\*\*P<0.001 vs control by Student's t test.

markedly lowered by 50% (P<0.001) at the Cu-R and by 55% and 48% (P<0.001) in Zn-

R and Vitamin-E R groups respectively. This reduction of placental activity of 11 β HSD is associated with a general decrease in placental protein concentration (Fig. 3B). The activities of AHH, MD ase, and PK ase in placenta were unchanged, thus unresponsive to maternal micronutrient restriction (Table-III). Activity of the glucocorticoid -inducible enzyme GS ase was elevated 1.3 fold, 1.4 fold and 1.4 fold respectively in Cu-R , Zn-R and Vitamin-E restricted group (P<0.001).

*Systolic blood pressure and lipid profiles*

At postnatal day 180 of age systolic blood pressure (Fig. 5), fasting triglyceride and total cholesterol (Table-IV) concentrations were significantly higher in offspring of dams fed the micronutrient restricted diet.

TABLE III : Placental enzyme activities of 11 β HSD, MD ase, AHH, GS ase and PK ase in control pregnant and micronutrient restricted (Cu-R or Zn-R or Vitamin-E R) pregnant mice on day 19 of gestation.

Biochemical profiles	Control	Cu-R	Zn-R	Vitamin-E R
11 β HSD units (n=7)	40±2.1	20±1.0***	18±1.8***	21±1.2***
MD ase units (n=7)	505±6.7	504±4.9	503±7.01	506±6.9
GS ase units (n=7)	60±3.1	80±2.9***	82±2.8***	86±2.2***
PKase units (n=7)	4.0±0.5	3.8±0.2	4.1±0.4	4.2±0.2
AHH ase units (n=7)	105±4.5	103±2.0	101±3.1	103±1.7

Values represent mean±s.d., n, number of observations. \*\*\*P<0.001 vs control; by Student's t test; Glutamine synthase (GSase), Aryl hydrocarbon hydroxylase (AHH), Malate dehydrogenase (MD ase), 11 β hydroxy steroid dehydrogenase (11 β HSD) and pyruvate kinase (PK ase); 11 β HSD units are percent conversion of corticosterone to 11 dehydrocorticosterone/10 min/mg protein. AHH, PKase, MDase and GSase units are nmoles product formed/mg protein.

TABLE IV : Plasma measurements of triglycerides and cholesterol levels in fasted 6 month-old offspring mice of Cu-R, Zn-R and Vitamin-E R groups and controls.

Biochemical profiles	Control	Cu-R	Zn-R	Vitamin-E R
Triglyceride (mg/dl) (n=8)	118±3.8	215±3.7***	208±5.8***	216±3.2***
Total cholesterol (mg/dl) (n=8)	200±5.8	300±4.0***	298±7.0***	306±5.2***

Values represent mean±s.d., n, number of observations. \*\*P<0.001 vs control; by Student's t test.



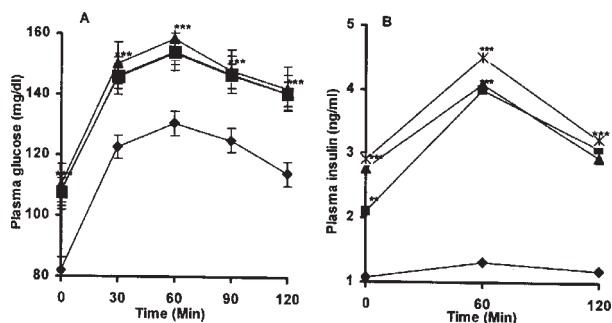


Fig. 4 : Blood glucose (A), insulin (B) levels during an intraperitoneal glucose tolerance test IPGTT in 6 months-old mice offspring of both MR (□ Cu-R; ▽ Zn-R; × Vitamin-E-R) and control (□) groups. Results are presented as mean±s.d., from n=8; \*\*\*P<0.001; \*\*P<0.01 vs control b Student's t test.

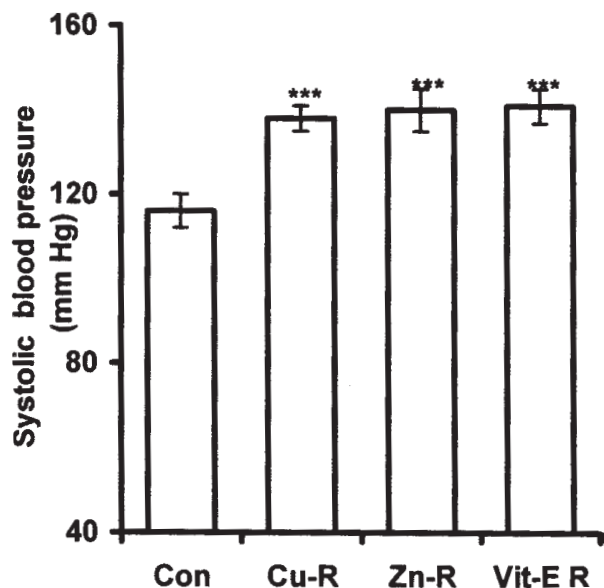


Fig. 5 : Systolic blood pressure in 6 months-old mice offspring of both micronutrient restricted (□ Cu-R; ▽ Zn-R; × Vitamin-E-R) and control (□) groups. Results are presented as mean±s.d., from n=8; \*\*\*P<0.001 vs control by Student's t test.

#### Glucose tolerance and insulin levels

The mean blood glucose results from the intraperitoneal glucose tolerance tests are shown in Fig. 4A. The zinc, copper and vitamin E restricted food given mice had significantly impaired glucose tolerance

curve than control group. Insulin level in response to intraperitoneal glucose challenge was highest in Vitamin-E restriction group, followed by copper and zinc restricted offspring (Fig. 4B). Glucose intolerance in association with hyperinsulinemia suggests the presence of insulin resistance in all the offspring of mineral (Cu or Zn) and vitamin E restricted groups.

#### DISCUSSION

The “thrifty phenotype hypothesis” (17) suggests that intrauterine growth retardation renders a growing fetus more susceptible to the development of insulin resistance syndrome in the adult, and that the time of onset and severity of the condition depends upon factors encountered in adult life such as nutritional status, stress, social class, smoking, alcohol and lack of exercise. The present study was performed to assess the role of maternal dietary micronutrient restriction (Cu or Zn or Vitamin-E) on the development of insulin resistance and hypertension in later life of the offspring.

In this study we observed that micronutrient deficiency in the diet during pregnancy had adverse effect upon the reproductive performance of mice and exerted gross effects upon litter size, fetal weight and crown rump length at birth. These findings are consistent with previous observations, which were reported with relation to macronutrient maternal insult during different time points of gestation periods (18–19). The present results emphasize on the relevance of micronutrients because their deficiency (Zn or Cu or Vitamin-E) during the gestation may influence the programming of birth weight, crown rump



length and litter size of offspring. In the present study, we demonstrated for the first time that chronic micronutrient deficiency (Zn or Cu or Vitamin-E) during pre-conception, conception and weaning in mice increased their systolic pressure and insulin resistance. In the study carried out by Langley and Jackson (20), maternal protein intake was restricted only during early half of pregnancy, and the offspring's born to mothers fed on protein restricted diet developed raised systolic blood pressure, suggesting that pregnancy period may be the critical period in which blood pressure is programmed. We also observed that maternal micronutrient restriction (Cu or Zn or Vitamin-E) caused a reduction in the placental 11  $\beta$  HSD-2 expression and activity. The role of this enzyme is to metabolize active cortisol (or corticosterone in rat) to inactive cortisone (11-dehydro cortisone in rat) (21) and, thus, serves to protect the fetus from the deleterious effects of excess level of active maternal glucocorticoids (22–23). The reduced placental 11  $\beta$  HSD-2 activity may be mediated by attenuating the levels of 11  $\beta$  HSD-2 gene transcription, suggesting a micronutrient/11  $\beta$  HSD-2 gene interaction. These findings are in accordance with the earlier observations in which elevated glucocorticoid, either by inhibiting 11  $\beta$  HSD-2 by carbenoxolone or by feeding the low protein diet during pregnancy (24) resulted in reduced fetal growth and programmed hypertension and insulin resistance in the offspring during later life (25).

The placental 11  $\beta$  HSD deficiency may form a common pathway where by maternal environmental factors, such as maternal micronutrition restriction, alter fetoplacental development and programme hypertension.

The molecular mechanisms through which maternal micronutrient restriction of either copper or zinc or Vitamin E restriction selectively alters placental 11  $\beta$  HSD activity remain to be determined. The maternal micronutrients restriction related reduction in placental 11  $\beta$  HSD activity is not merely part of an overall attenuation of placental function, because MD ase and PK ase activity (except total protein content) remain unaltered, while GS ase activity increased. Indeed, elevated placental GS ase may reflect increased glucocorticoid action (26) within the placenta as a consequence of inactivation by 11  $\beta$  HSD-2.

Poor nutrition in early life is associated with increased risk of type-2 diabetes and the insulin resistance syndrome in later life (27). Maternal macronutrient deficiency may affect fetal growth and development directly through the availability of nutrients for transfer to the fetus and also permanently alter glucose/insulin metabolism (28). Several metabolic abnormalities lead to insulin resistance in the offspring of rat dams fed a protein-restricted diet. The organ weights of muscle and liver were reduced (29). The activities and gene expression of insulin-sensitive hepatic enzymes were changed. In addition, the glucokinase activity was reduced; and phosphoenolpyruvate carboxykinase and hepatic glucose production increased (30). Recently, it was reported that phosphatidylinositol-3 kinase activation in response to insulin was impaired in adipocytes from 15-month old offspring of rat dams fed on protein restricted diet (31).

The question is whether a link exists between poor maternal micronutrient deficiency and a predisposition to insulin

resistance and hypertension in later life. An impaired glucose tolerance was observed in offsprings born to dams fed either the zinc or copper or vitamin E restricted diet during the weaning and pregnancy period. The insulin response peak to intraperitoneal glucose challenge was greatest in vitamin E restricted diet fed group followed by copper and zinc restrictions respectively. These findings suggest that the micronutritional deficiency in early life predisposes the offspring to insulin resistance syndrome in later life. The chronic maternal micronutrient deficiency (Zn or Cu or Vitamin-E) modulates the expression of placental 11  $\beta$  hydroxysteroid dehydrogenase-2 and *per se* predisposes the offspring to insulin resistance and hypertension in later life. It may be inferred that the feeding of micronutrient restriction diet during

conception and early life may overexpose the fetus to imprinting effects of maternal glucocorticoids. This finding suggests that the hypertension and insulin resistance are independent and may operate through different mechanisms. Further studies will be required to elucidate the molecular physiological mechanism by which chronic micronutrient deficiency predispose the offspring to insulin resistance and hypertension in later life.

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