Original Paper

Effect of Folic Acid Supplementation on Renal Phenotype and Epigenotype in Early Weanling Intrauterine Growth Retarded Rats

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Key Words

Intrauterine growth retardation • p53 methylation • Glomerular volume • Hypertension

Abstract

Background/Aims: The objective of this study was to examine the responses of p53 promoter methylation involved in kidney structure and function of early weaning intrauterine growth retarded (IUGR) rats to dietary folic acid supplementation. **Method:** Sprague-Dawley rats were fed isocaloric diets containing either 21% protein diet (normal feed) or 10% protein diet throughout pregnancy and normal feed during lactation. After weaning, Offspring were then fed onto normal feed and normal feed supplemented with 5 mg folic acid/kg feed for a month, this produced 4 dietary groups (maternal diet/ weanling diet): Con, Folic, IUGR and IUGR+Folic. Renal function, renal structure, p53 promoter methylation and protein expression of offspring rats were measured at postnatal 2 months and 3 months. **Results:** Glomerular volume, blood urea nitrogen, 24 hours urine protein were significantly elevated in IUGR rats compared with Con rats but were decreased by dietary folic acid supplementation. p53 protein expression in IUGR rats were significantly higher than that in Con rats, and p53 promoter methylation status in IUGR rats was reduced significantly compared with Con rats. However, the changes in p53 gene expression and DNA methylation status of IUGR rats were reversed by dietary folic acid supplementation. **Conclusions:** Our study showed for the first time that folic acid supplementation during early period of life could reverse the abnormality in renal p53 methylation status and protein expression, glomerular volume and renal function of IUGR rats offspring.

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Introduction

BARKER'S FETAL ORIGINAL ADULT DISEASE hypothesis proposes that a deprived intrauterine milieu leads to permanent changes in cellular biology and systemic physiology.

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Early stigmata of this phenotype include intrauterine growth retardation (IUGR). Several epidemiological studies closely linked IUGR to adult onset diseases such as hypertension [1-4]. Our previous rats study showed that low birth weight offspring of maternal protein restriction diet exposure decreased glomerular number and impaired renal function, and caused hypertension from postnatal 2 months old [5]. However, the basic mechanism that IUGR offspring led to later renal dysfunction was not fully uncovered. Supplementation of the maternal protein-restricted (PR) [6] diet with folic acid or glycine prevents induction of hypertension and endothelial dysfunction in the offspring [7], indicating that 1-carbon metabolism is central to the mechanism underlying induction of an altered phenotype. However, most dietary folic acid intervention was implemented during intrauterine life [8-10], very few studies have address the question that whether folic acid supplementation in early postnatal life could also be a nutritional strategy for combating IUGR, especially reverse the renal dysfunction by maternal PR diet.

p53 is best known as a tumor suppressor that regulates cell-cycle, differentiation, and apoptosis pathways and is required for early events of metanephric development [11-13]. Our previous rats work showed that the p53 protein expression was down-regulation in low birth weight offspring kidney (under publishment). However, the deep molecular mechanisms responsible for the reduction were unknown. This study was to investigate the role of p53 methylation in maternal malnutrition programming further, and test whether dietary folic acid supplementation during early life could alleviate the negative effects that IUGR confers upon renal phenotype and p53 methylation profile in the rat offspring.

Materials and Methods

Rats and experimental design

The experimental protocols were approved by the Experimental Animal Center of the Second Xiang Ya Hospital of Central South University. Totally 16 male and 16 female three-month-old Sprague-Dawley (SD) rats (weighting 250-300 g) were involved in this study. They were acclimatized to laboratory conditions for 7 days, and then the male and female rats were placed in individual cages with a ratio of 1:1. When a vaginal plug appeared, that day was designated as gestation day 1. Pregnant rats were randomly divided into IUGR group (IUGR) and control group (Con). The IUGR model was modified from our previous paper [5]. In IUGR, the pregnant rats were fed 10% protein diet (protein restriction feed) only during pregnancy, and 21% protein diet (normal feed) during lactation. The pregnant rats in Con were fed normal feed during pregnancy and lactation. At weaning, offspring of two groups were randomly assigned to normal feed or normal feed supplemented with 5 mg folic acid/kg feed for a month after weaning, which was specifically selected to represent the juvenile-pubertal period. This produced 4 dietary groups (maternal diet/weanling diet): Con, Folic, IUGR, and IUGR+Folic. The dose of folic acid used in the study was according to the observations in earlier reports [9, 14]. From postnatal 2 months, all of rats were given normal feed until postnatal 3 months.

The animals were housed in a room with a controlled temperature and a 12-h light/dark cycle. The composition of diet was described in our previous study [5]. The animals were allowed free access to water and feed. There were 8-10 offspring in every nest during lactation, excluding the nest with more than 14 offspring or less than 8. When the offspring were weaning at postnatal 21 days, all of the rats were marked, and the serial number was divided into 8 rats per group according to random number table. Then the random group rats entered the subsequent study at every time point. The method is consistent to the previous studies [15, 16]. Twenty-four hours urine sample, blood samples and tissue samples were collected at the age of 21 days, 2 months and 3 months. Postnatal 21 days was weaning time. Postnatal 2 months was intervention terminal time. Postnatal 3 months was specially chosen because it is time point of rats adulthood and adult disease emergence based on our previous work [5]. The kidney samples were taken within 5 min after sacrifice. A half of right kidney was snap frozen in liquid nitrogen before being stored at -80°C, and a half of right kidney was stored in 4% paraformaldehyde for histology study. The measurement method of serum creatinine (Scr), blood urea nitrogen (BUN), 24 h urine protein and glomerular number was detailed described in our previous paper [5].

Determination of Glomerular Volume

Glomerular volume was determined according to the method described by Manalich et al [17]. The kidneys were bisected and immersed in 4% paraformaldehyde. After 10 days, halves of the kidney samples were cut into slices 4 mm thick. Histomorphometric analyses were done by a renal pathologist who was blinded as to the origin of the biopsy. After stained with hematoxylin and eosin, analyses were done with the help of a computerized area measurement system supported by Windows XP that includes capture of the image from an Nikon Eclipse E400 microscope and digitalization of this image (SONY DSC-W55). 20 glomeruli of every slice were selected to glomerular area (A). The glomerular volume was calculated with the use of the following formula:

$$G = ((\beta/K) \times A)^{3/2}$$

Where $\beta = 1.38$ is the shape coefficient of spheres, and $k = 1.1$ the size distribution coefficient. A is the glomerular size.

Western blotting

Homogenates from renal were prepared in 400 μ l of RIPA lysis buffer (Applygen, China) using a Dounce homogenizer. Homogenates were incubated for 1 h at 4 °C and centrifuged at 10,000 $\times g$ for 30 min at 4 °C. Protein in the supernatants was measured using the BCA protein quantitative kit (well biology, china). For immunoblotting, proteins (30–60 μ g) were separated by 12.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blots were incubated with rabbit polyclonal anti-rats p53 (1:3000) antibodies (CST, USA). The primary antibodies were detected using horse radish peroxidase-conjugated goat anti-rabbit IgG (1:1000) antibodies (BOSTER, china). Bands were visualized by enhanced chemiluminescence (Applygen, China).

Bisulfite Modification and Bisulfite Genomic Sequencing

Genomic DNA was isolated using the AURAGENE Genomic DNA Purification Kit (AURAGENE, China) according to the manufacturer's protocol. The size of DNA estimated by agarose-gel electro-phoresis was >10 kb in all instances. The 8 genomic DNA of every group were mixed together. The methylation status of individual CpG sites within the promoter region of the p53 gene was determined by the sodium bisulfite-sequencing assay using the EZ DNA Methylation-Gold™ Kit (ZYMO RESEARCH, USA) according to the manufacturer's protocol. In the DNA bisulfite modification, unmethylated cytosine is converted to uracil, whereas methylated cytosine remains unchanged. The following are PCR primers, p53-BSP-F: TAATTAAAAGTAAATAGA; p53-BSP-R: ATCTTATTTCAAATAC (BGI, China) which amplify a 365 bp product. The PCR conditions were: denaturation at 94°C for 4 min, 35 cycles at 94°C for 30s, 56°C for 30s and 72°C for 30s. The purified PCR products were cloned into pMD18-T Vector using the pMD18-T Vector (Takara, Japan) according to the manufacturer's protocol. 20 positive clones of every group were sequenced.

Statistical Analyses

Data were analyzed using SPSS version 20.0 (SPSS, Inc, Chicago, IL, USA). All results were shown in the form of mean $\pm$ standard deviation (mean $\pm$ SD). Student's t-test was performed to detect differences in means between the two groups and chi-square (χ^2) test for proportion differences. Statistical significance of the differences between four groups was determined by two-way ANOVA, followed by LSD-t test. A P value less than 0.05 was considered statistically significant.

Results

IUGR Model Building

16 female rats became pregnant after continuing circular mating for 14 days. All pregnant rats labored during gestation day 20–23, without miscarriage. Table 1 shows the baseline characteristics of the Con and IUGR during perinatal period. There was no difference in the average litter size between the groups. The body weight of offspring was significantly lower in IUGR (5.59 $\pm$ 0.55 g) compared to Con (6.95 $\pm$ 0.64 g) ($p < 0.01$), which was accompanied with higher incidence of low birth weight offspring rats.

Table 1. Baseline characteristics of the study groups in perinatal period

	Pregnant rat number	Total offspring number	Mean number of Offspring per litter ^a	Average birth weight of offspring rats (g) ^a	Number of LBW offspring rats (%) ^b	Perinatal mortality (%)
Con	8	86	10.75±1.58	6.95±0.64	3(3.49)	4(4.65)
IUGR	8	92	11.50±0.93	5.59±0.55**	70(76.09) **	8(8.70)

^aValues are presented as means ±SD. ^bLBW (low birth weight) was defined as less than average birth weight of offspring rats in the control group minus two standard deviations. **p < 0.01 compared with control group

Growth after birth

Table 2 presents the baseline data between IUGR and Con, there is no difference with body weight, kidney weight in postnatal 21 days. Table 3 presents the comparison of body weight and kidney weight in four groups at

Table 2. Baseline characteristics of the study groups in postnatal 21 days

	Con	IUGR	t	P
Body weight (g)	36.73±5.21	33.27±9.26	0.923	0.372
Kidney weight (g)	0.20±0.02	0.18±0.02	1.790	0.095
BUN (mmol/l)	6.82±1.03	6.68±0.88	0.292	0.775
Scr (umol/l)	21.39±3.89	24.18±4.58	1.317	0.209
24 h urine protein (mg/kg·day)	25.92±4.57	26.24±5.14	0.133	0.896
Glomerular Volume (um ³ ×10 ⁵)	52.94±8.68	55.88±10.04	0.630	0.540

Data are given as mean ± SD.

postnatal 2 months and 3 months. There is no difference with body weight and kidney weight at any time point between Con and Folic. Comparing with the offspring in Con, the body weight and the kidney weight were significantly lower in IUGR at postnatal 2 months, while there was no difference at postnatal 3 months. The offspring rats in IUGR exhibited stage catch-up growth after weaning, which agreed well with the previous report [5]. The body weight and kidney weight of IUGR+Folic at postnatal 2 months is lower than the Con. While body weight of IUGR+Folic at postnatal 3 months was lower than Con, but the kidney weight reached Con (for details, see in table 2).

Renal Function

The baseline data with renal function (Scr, BUN, 24 h urine protein) between IUGR and Con was not different in postnatal 21 days (see in table 2). The comparison of Scr, BUN and 24 h urine protein at postnatal 2 months and 3 months between the 4 groups is shown in table 3. There was no difference in Scr between the four groups at the age of 2 months and 3 months, and there was no difference in BUN and 24 h urine protein between the four groups at the age of 2 months. However, BUN and 24 h urine protein of the IUGR was higher than the Con (p < 0.05) at 3 months of age. The renal function can be rescued by folic acid supplementation, BUN and 24 h urine protein was lower in IUGR+Folic than IUGR (p < 0.05) at 3 months of age.

Glomerular Number and Volume

In IUGR pups, maternal protein restriction diets significantly reduced offspring glomeruli per kidney section to 22187±998 at postnatal 2 months and 22437±1015 at postnatal 3 months (P < 0.01). In contrast, control pups contained glomeruli per kidney section 28875±1061 at postnatal 2 months and 29000±964 at postnatal 3 months. When adding folic acid into the feed, the glomeruli number remained similar between postnatal 2 months and postnatal 3 months (22060±1266 and 22250±886 respectively). There was no difference with glomerular number between IUGR and IUGR+Folic at any time point.

Table 2 presents the baseline glomerular volume data between IUGR and Con, there is no difference in postnatal 21 days. The glomerular volume of the four groups was not different at postnatal 2 months. However, the glomerular volume of IUGR was significantly larger than Con at postnatal 3 months. When adding folic acid, the glomerular volume of IUGR+Folic became smaller comparing with IUGR (100.13±16.07 vs 116.80±13.46, P<0.01) (for details, see in table 2 and 3).

Table 3. Comparison of the parameters of offspring between the four groups (n=8)

	Con	Folic	IUGR	IUGR+Folic	F	P
Body weight (g)						
2m	173.70±38.96	162.16±29.99	123.69±16.80 ^b	121.74±14.28 ^b	7.761	0.001
3m	265.97±19.38	254.14±16.77	270.37±21.60	241.16±20.27 ^{ad}	3.581	0.026
Kidney weight (g)						
2m	0.90±0.1088	0.83±0.1280	0.71±0.08 ^b	0.83±0.12 ^c	3.26	0.025
3m	0.97±0.0952	0.95±0.0894	1.03±0.12	0.97±0.0948	0.928	0.440
BUN (mmol/l)						
2m	7.27±0.79	7.06±0.86	7.17±0.82	7.22±0.76	0.102	0.958
3m	7.03±0.98	7.15±0.71	8.51±1.04 ^b	7.32±1.07 ^c	4.025	0.017
Scr (umol/l)						
2m	24.93±5.08	23.96±4.55	26.27±4.34	25.60±4.91	0.348	0.791
3m	27.07±4.53	25.91±4.89	29.28±3.41	27.42±3.83	0.886	0.461
24 h urine protein (mg/kg·day)						
2m	96.70±9.42	97.20±7.78	100.39±12.29	101.95±10.27	0.504	0.683
3m	93.18±11.26	92.32±9.39	114.78±13.48 ^b	98.81±12.66 ^c	6.228	0.002
Glomerular Number						
2m	28875±1061	28437±1237	22187±998 ^b	22060±1266 ^b	86.81	0.000
3m	29000±964	29312±843	22437±1015 ^b	22250±886 ^b	143.47	0.000
Glomerular Volume (um³×10⁵)						
2m	74.14±12.86	75.77±12.12	79.65±14.76	78.35±10.53	0.309	0.819
3m	89.75±15.68	86.37±16.84	116.80±13.46 ^b	100.13±16.07 ^{a,c}	7.784	0.001
p53 protein expression						
2m	0.31±0.07	0.28±0.05	0.48±0.05 ^b	0.39±0.08 ^{ac}	16.89	0.000
3m	0.16±0.032	0.17±0.04	0.37±0.02 ^b	0.27±0.02 ^{bd}	96.48	0.000
p53 promoter methylation						
3m	17.25±0.64	17.05±0.61	3.55±0.67 ^b	8.10±0.79 ^{bd}	1981.32	0.000

Data are given as mean ± SD. BUN: blood urea nitrogen. Scr: serum creatinine. 24 h urine protein: 24 hour urine protein. ^ap < 0.05 compared with control group, ^bp < 0.01 compared with control group. ^cp < 0.05 compared with IUGR group, ^dp < 0.01 compared with IUGR group.

p53 protein expression and p53 promoter methylation

The p53 protein expression was gradually decreased from postnatal d 1 to postnatal 3 months in both Con and IUGR (data are not shown). However, maternal protein restriction diets significantly made the p53 protein expression of IUGR higher than Con at postnatal 2 and 3 months. When the IUGR offspring adding with folic acid, the p53 protein expression of IUGR+Folic became less than IUGR, although it is still more than Con at postnatal 2 and 3 months (table 3 and figure 1). Totally, there are 18 CpG islets in the p53 promoter. The maternal diet and folic acid supplementation affected renal p53 promoter methylation. p53 promoter methylation was significantly lower in IUGR than Con offspring, but p53 promoter methylation was greater in IUGR+Folic than IUGR offspring at postnatal 3 months (table 3 and figure 2).

Discussion

In the present study, our study demonstrated that dietary folic acid supplementation during early weaning period prevented the abnormality in p53 methylation status and renal function in IUGR rats kidney by maternal protein restriction diets. Our study implicated that folic acid supplementation in weaning IUGR rat could intervene maternal malnutrition programming successful: maternal low protein diet exposure-offspring renal p53 gene epigenotype abnormalities-offspring glomerular volume abnormalities-offspring renal dys-

function-offspring adult onset hypertension.

The glomerular number was decreased, BUN and 24 hour urine protein were increased in IUGR rats, which agreed well with our previous report [5] and indicated our IUGR rats model is stable. Glomerular is the base of renal function and the most components of nephron. There is an inverse relationship between nephron number and blood pressure, irrespective of whether nephron number is reduced congenitally or in postnatal life (as from partial renal ablation or acquired renal disease). Brelmer et al.'s study [18] proposed that congenital nephron number decrease is one of the important mechanisms of hypertension and renal injury. The decreasing glomerular number in IUGR coincided well with several animal study which showed that the nephron number of IUGR is decreased 15-60% and was an independent risk factor for hypertension in adulthood [19].

p53 function is required for early events of metanephric development [11-13]. In the present study, promoter methylation status of p53 was significantly decreased by 80%. Studies have shown that the glomerular cell apoptosis [20] and podocyte apoptosis [21] is an important reason of kidney disease. Wild-type p53 transgenic mice exhibited that p53 transgenic kidneys grew to only half of their expected size and contained about half of the normal number of nephrons because of the loss of uncom-

mitted mesenchymal cells by apoptosis [11]. Simon et al. research [22] showed protein restriction in pregnancy is associated with increased apoptosis of mesenchymal cells at the start of offspring rat metanephrogenesis. Pham et al. research [15] showed that uteroplacental insufficiency reduced p53 CpG methylation and affected mRNA levels of key apoptosis-related proteins (Bcl-2/Bax) in the kidney. Moreover, Godley et al.'s wild-type p53 transgenic mice showed that glomeruli volume was compensatory hypertrophy [23]. Decrease of glomerular

Fig 1. Comparison of the p53 protein relative expression between four groups at postnatal 2 and 3 months. Data are given as mean $\pm$ SD. The p53 protein relative expression is the ratio of p53 protein band gray value to β -actin protein band gray value. Con group in 2m was translated to 1, and other group data was translated accordingly. ^a $p < 0.05$ compared with control group, ^b $p < 0.01$ compared with control group, ^c $p < 0.05$ compared with IUGR group, ^d $p < 0.01$ compared with IUGR group.

Fig 2. Comparison of the p53 promoter methylation between four groups at postnatal 3 months. Data are given as mean $\pm$ SD. ^a $p < 0.05$ compared with control group, ^b $p < 0.01$ compared with control group, ^c $p < 0.05$ compared with IUGR group, ^d $p < 0.01$ compared with IUGR group.

number led to compensatory hypertrophy, high perfusion, or sclerosing necrosis of nephron, which result in hypertension [20]. Therefore, we deduced that maternal protein restriction during pregnancy altered offspring rats p53 DNA CpG methylation, increased glomerular cell apoptosis or podocyte apoptosis, reduced glomeruli number. Decrease of glomerular number led to glomerular volume compensatory hypertrophy, as the glomerular volume was increased in the IUGR rat kidney.

Abnormal one-carbon metabolism and subsequent DNA methylation abnormality were the most possible mechanisms responsible for the persistently negative effects of IUGR on the offspring. There is some information that changes in phenotype induced by nutritional constraint during early life can be reversed by effective intervention. Previous studies in rats emphasized the usage of folic acid supplementation to rescue the impaired hepatic DNA methylation and genes expressions of the offspring by maternal malnutrition during gestation period [9, 10]. Besides gestational stage, Juvenile-pubertal period is another highly plastic stage and intervention period [14, 16]. In this study, we supplemented IUGR rats with folic acid in Juvenile-pubertal period. We observed that p53 promoter methylation status was reversed in IUGR+Folic rats comparing with IUGR rats in postnatal 3 month. The up-regulated p53 promoter methylation status was responsible for the decreased p53 protein expressions in IUGR+Folic rats. Kidney completes organ development before gestational week 36 in uterus and nephron number decreases gradually after birth. Therefore, this could be a reason for ineffectiveness of folic acid supplementation to rescue nephron numbers. However, it is worthy noting that the change in p53 promoter methylation and protein expressions were paralleled by a decrease in kidney glomerular volume, BUN and 24 hour urine protein in IUGR+Folic, suggesting that folic acid supplementation prevented glomerular compensatory hypertrophy and improved function through epigenetic pathway. The folic acid supplementation intervention effect coincided well with the studies using rats and piglets for the nutritional programming intervention experiments [14, 16].

Conclusion

Our results suggested that dietary folic acid supplementation during early weaning period reversed the abnormality in renal p53 methylation status and protein expression, glomerular volume and renal function of IUGR rats offspring. Our study provides insight to ameliorating the negative effects of IUGR on the offspring by nutritional strategy during early period of life.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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