Journal of Animal & Plant Sciences, 2010. Vol. 7, Issue 3: 852- 859.
Publication date: 27/07/2010, *http://www.biosciences.elewa.org/JAPS;* **ISSN 2071 - 7024**

Effects of maternal folic acid supplementation on gilts reproductive performance and apoptosis-related gene expressions of kidney in newborn piglets

Jingbo Liu, Ying Yao, Bing Yu and Daiwen Chen*

Key Laboratory for Animal Disease Resistance Nutrition of the Ministry of Education of China, Institute of Animal Nutrition, Sichuan Agricultural University, Xinkang Road 46, Ya'an, Sichuan Province, P R China, 625014

**Corresponding author: Email chendwz@sicau.edu.cn; Phone Number: 86-835-2885106*

Key words
Folic acid; intrauterine growth restriction; gilts; gene expression, kidney

1 SUMMARY

Intrauterine growth retardation (IUGR) in piglets was associated with DNA methylation in the promoter of renal p53 gene. The effect of maternal folic acid supplementation was investigated on kidney mRNA expression of DNA methyltransferase1 (DNMT-1), and apoptosis-related gene expressions between intrauterine growth restriction（IUGR）and normal body weight（NBW）piglets. Gilts were randomly allocated to two diets of control (C, folic acid 1.3mg/kg) or folic acid supplementation (F, folic acid 30mg/kg) diets with 12 replicates of 1 gilt each after mating. The reproductive traits of gilts were recorded and kidney samples were collected to detect gene expressions from newborn piglets of NBW and IUGR (n=8 piglets NBW and IUGR per treatment, respectively). Serum folic acid concentrations of gilts and piglets were both greater in folic acid supplementation group (*P*<0.01), but there were no significant differences in reproductive performance. IUGR contributed to decreased DNMT-1, Bcl-2 and IGF-1 expressions, but up-regulated Bax and p53 gene expressions. Maternal folic acid supplementation reversed Bcl-2, p53 and DNMT-1 expressions. Collectively, maternal folic acid supplementation reversed IUGR altered kidney expression levels of genes related to one-carbon metabolism and apoptosis, thus providing a chance to rescue the phenotype of IUGR individuals with folic acid.

2 INTRODUCTION

There is growing evidence that impaired intrauterine growth and development, termed intrauterine growth retardation (IUGR), could negatively program the postnatal growth and health status, characterized by reduced neonatal survival, low nutrient utilization, poor carcass

traits and compromised long term health (Wu et al., 2006). The decreased postnatal performance could be likely attributed to altered phenotype as described previously by Wang et al. (2008) who identified differentially expressed proteins in relation to energy metabolism, protein turnover, immune response and cellular signaling between pigs with normal birth weight and IUGR. Additionally, IUGR was found to be associated with altered one-carbon metabolism and DNA methylation status (MacLennan et al. 2004), which might exert permanent influence on phenotype and heath status(MacLennan et al., 2004). Previous studies found that fetus experiencing IUGR could negatively influence the promoter methylation status of kidney p53 and hence its mRNA expression to regulate apoptosis-related factor Bcl-2 and Bax (Pham et al., 2003), which plays an important role in cell death, with evidence that altered Bcl-2 expression could also be associated with insulin like growth factor-1 (IGF-1)(Gobe et al., 2000), a critical factor in regulating cell turnover.

Upon realization of the negative effects induced by IUGR, it was suggested that maternal folic acid could be considered as one part of the potential strategies to prevent the negative effects of maternal constraints on fetal development (MacLennan et al., 2004; Xu et al., 2007). Therefore, we hypothesized that kidney gene expressions could be negatively influenced by IUGR, and tested whether the changed kidney gene expressions of newborn IUGR piglets could be reversed after maternal folic acid supplementation.

3 MATERIALS AND METHODS

The experimental procedures observed all animal protection requirements and were approved by the University of Sichuan Agricultural Animal Care Advisory Committee.

3.1 Animal and diets: Twenty four Yorkshire gilts were allocated to two diets(Table 1): control diet (C; basal diet +1.3mg/kg folic acid), and folic acid supplementation diets (F; basal diet+30mg/kg folic acid) immediately after mating and continued on these diets from conception until delivery. The diet formulation was based on corn-and soybean meal (Table 1) to meet or exceed the nutrient requirement (NRC, 1998). Gilts were housed in individual feeding stalls in a breeding facility, feed intake was 2 kg/d from day 0 to day 84 of pregnancy, and 2.8 kg/d from day 85 to day 114 of pregnancy.

Table 1: Dietary composition of the basal diet[1]

Ingredients, %	Folic acid level (mg/kg folic acid)	
	1.3	30
Corn	72	72
Soybean meal(44%,CP)	22	22
Fish meal	2	2
Calcium phosphate	1.4	1.4
Calcium carbonate	1.6	1.6
Salt	0.4	0.4
Choline chloride	0.1	0.1

Vitamin premix[2]	0.3	0.3
Mineral premix[3]	0.2	0.2
Folic acid supplementation (mg/kg folic acid)	1.3	30

[1]The calculated composition for DE, CP, lysine, Ca and P of the basal diet were 13.8 MJ/kg, 17.2%, 0.9%, 1.2% and 0.7%, respectively. The added folic acid was pteroylglutamicacid, C19H19N7O6.

[2]Provided per kilogram of diet: vitamin A, 12,800 IU; vitamin D3,2,600 IU; vitamin E,44 IU; menadione,4 mg; thiamin,2.4 mg; riboflavin,8.8 mg; pyridoxine,3.2 mg; vitamin B12,0.028 mg; niacin,32 mg; pantothenic acid,24 mg; biotin,0.5mg.

[3]Provided per kilogram of diet: Fe as ferrous sulfate 160 mg; Cu as cupric sulfate 30 mg; Zn as zinc sulfate 160 mg; Mn as manganous sulfate 30 mg; Se as sodium selenite 0.5 mg; I as potassium iodide 0.6 mg.

Table 3: Primer sequences of target and house keeping genes

Gene	Primer sequences (5'-3')	Product size(bp)	Genebank NO.
Bcl-2	Left: CTGGTGGTTGACTTTCTCTCCT Right: TAGGGGTTTCCGCTTCTGAT	124	NM214285
P53	Left: CACTGGATGGCGAGTATTTCAC Right: CTTAGACTTCAGGTGGCTGGA	152	AF098067
Bax	Left: GGTCGCGCTTTTCTACTTTG Right: CGATCTCGAAGGAAGTCCAG	111	AJ606301.1
IGF-1	Left: CTGAGGAGGCTGGAGATGACT Right: CGATCTCGAAGGAAGTCCAG	136	DQ121132
DNMT-1	Left: AGGTGAGGACATGCAGCTTT Right: AACTTGTTGTCCTCCGTTGG	213	BF198895
H2A	Left: TGGATGTCCTTGGGCATG Right: AGATCCGGCGCTACCAGA	226	BP459633

3.2 Sample collection: Dams were selected from each group to collect peripheral blood (5 ml) by acute jugular venipuncture on day 60 of pregnancy. Immediately after delivery, the litter size, litter size born alive, stillborn, birth weight and weaning weight on d 20 of lactation were determined. Finally, a total of 16 newborn IUGR piglets (0.8-0.9 kg) were obtained from group fed diet C (8 IUGR piglets) and F (8 IUGR piglets) , and additional 16 NBW (neonatal piglets with average birth weight) were obtained from C (8 IUGR piglets) and FS (8 IUGR piglets) group, respectively. IUGR piglets was selected based on body weight less than 66% of the average litter weight, and in fact, the bodyweight of the IUGR piglets was about 60% of the average litter weight (Average litter weight in C and FS were 1.35 and 1.37 kg, respectively). Blood samples were collected from the newborn piglets via anterior vena cava puncture into 5-mL heparin-free glass tubes. Blood samples from dams and newborn piglets were centrifuged at 3,000 rpm for 10 min to collect serum and then immediately stored at -20C for later analysis. Selected piglets were killed by jugular puncture after anesthesia and

the kidney samples were collected and placed in liquid nitrogen and stored at -80C. Notably, IUGR piglets was defined when bodyweight of piglets less than 66% of the average litter weight, and in fact, the bodyweight of the IUGR piglets was about 60% of the average litter weight (Average litter weight in C and FS was 1.35 and 1.37 kg, respectively).

3.3 Reverse transcription polymerase chain reaction (RT-PCR) analysis for kidney genes: Total RNA was extracted using TRIzol Reagent (Tiangen) according to the manufacture's recommendations. The RNA was purified using an Rneasy Mini Kit (Takara) and treated with DNase (Takara) to remove contaminants. After testing the 5sRNA, 18sRNA and 28sRNA for integrity and excluding possible contaminants, the RNA were reverse transcribed using the Superscript First-strand Synthesis System for RT-PCR (Takara) following the manufacturer's protocols. Primers (Invitrogen) for target and house-keepting genes are shown in Table 2. Forty cycles of PCR amplification were performed on the CHRMO4-TM Thermal Cycler (BIO-RAD, Inc.) as follows: 95 degree centigrade (C) for 10 seconds, followed by 40 cycles of 95 C for 5 seconds, 60C for 25 seconds, melt curve conditions were 95C for 0 second, 65C for 30 seconds and 95C for 0 second (temperature change velocity: 0.5C/second).

3.4 Statistical analysis: The data were analyzed by GLM (SAS 8.0) to determine statistical differences between each group. After real-time reserve transcription-PCR amplification of ob-R, DNMT-1, GR, PPAR, AOX, MAT, CBS and MTHFR and normalization with H2A, the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expressions of each gene in the kidney. Differences were declared significant at $P<0.05$. Results are expressed as means ± SD.

4 RESULTS

4.1 Serum folic acid concentration in dams and piglets: The effects of maternal folic acid supplementation on serum folic acid concentrations in dams and piglets are presented in table 3. The serum folic acid concentrations in dams on day 60 of pregnancy were significantly increased by folic acid supplementation ($P<0.01$).

Table 3: Effects of maternal folic acid supplementation on serum folic acid concentrations in dams and piglets[1,2,3].

Source of serum		Folic acid supplementations(mg/kg)	
		C (1.3 mg/kg)	FS (30 mg/kg)
Dams(n=12)		113.73±8.96^{B}	187.18±6.21^{A}
Piglets	NBW (n=8)	19.24±1.26^{B}	27.30±1.58^{A}
	IUGR (n=8)	18.23±1.65^{B}	25.65±1.81^{A}

[1] Data are presented as Mean ± SD, different capital superscripts indicate statistically significant difference ($P<0.01$).

[2] C denotes control diet with folic acid supplementation of 1.3 mg/kg, and FS denotes FS diet with folic acid supplementation of 30 mg/kg; [3] NBW denotes data from piglets with average bodyweight, IUGR denotes intrauterine growth retardation.

Journal of Animal & Plant Sciences, 2010. Vol. 7, Issue 3: 852- 859.
Publication date: 27/07/2010, *http://www.biosciences.elewa.org/JAPS;* **ISSN 2071 - 7024**

4.2 Reproductive traits: The effects of maternal folic acid supplementation on reproductive traits of gilts are presented in table 4. The birth weight, litter size, litter size alive, average birth weight and average weaning weight were not affected by maternal folic acid supplementation (P>0.05).

4.3 Kidney gene expressions in response to folic acid supplementation and IUGR: Effects of IUGR and maternal folic acid supplementation on relative gene expressions of kidney are presented in Fig. 1. IUGR significantly down-regulated Bcl-2, IGF-1 and DNMT-1 expressions, and up-regulated Bax and P53 expressions compared to piglets of NBW (P<0.05; P<0.01). Maternal folic acid supplementation reversed Bcl-2, P53 and DNMT-1 expressions of IUGR piglets compared to those under control diets with no folate supplementation (P<0.05). Maternal folic acid supplementation had no effects on IGF-1 and Bax expressions (P>0.05).

Table 4: Effects of maternal folic acid supplementation on reproductive performance in gilts[1,2].

Parameter	Folic acid supplementation level	
	C (1.3 mg/kg)	FS (30 mg/kg)
Average birth weight (kg)	1.35±0.12	1.37±0.08
Litter size(n)	10.5±1.1	11.0±0.8
Litter size alive(n)	9.6±1.0	9.8±0.6
Pre-weaning survival (%)	100	98.6
Weaning weight (kg)	6.21±0.22	6.37±0.30

[1] Data are presented as Mean ± SD

[2] C denotes control diet with folic acid supplementation of 1.3 mg/kg, and FS denotes FS diet with folic acid supplementation of 30 mg/kg.

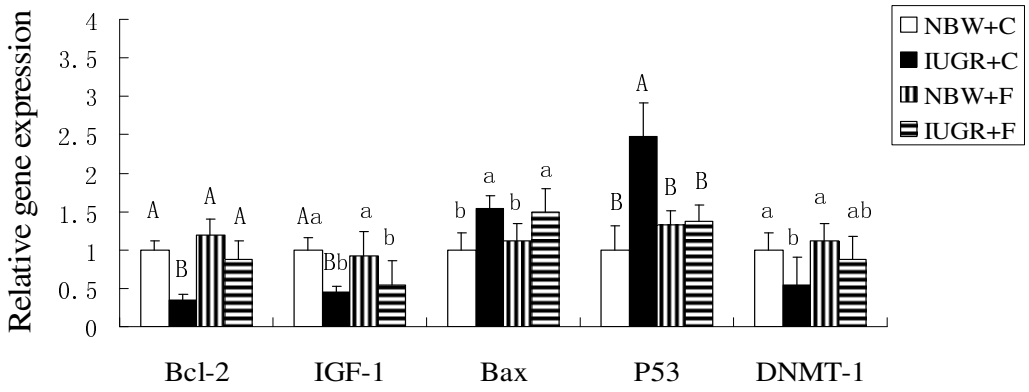

Figure 1: Relative gene expressions of DNMT-1, p53, Bax, Bcl-2 and IGF-1 in kidney. Different small and capital letters denotes significant differences at P < 0.05 and P<0.01, respectively.

5 DISCUSSION

Intrauterine growth retardation caused profound effects on kidney development as

previously reported (Hinchliffe et al., 2005). Further studies demonstrated that the mechanism mediating the negative effects of IUGR on kidney development could be associated with altered p53 DNA CpG methylation and expressions of key apoptosis-related proteins and increased renal apoptosis(Pham et al., 2003). However, we demonstrated maternal folic supplementation reduced the negative effects on kidney gene expressions related to cell apoptosis induced by uteroplacental insufficiency.

The present study demonstrated that maternal folic acid supplementation contributed to elevated circulating folic acid concentrations in neonatal piglets and dams, but no effects on reproductive traits were observed. This might be due to the sufficient folic acid levels after supplementation as suggested by NRC (1998), and the primiparous sow used in the present study could be affected by folic acid to a less extent compared to mutiparous sows (Lindemann & Kornegay, 1989). Pigs mostly have naturally occurring IUGR corresponding directly to litter size since the growth of a fetal pig has been shown to depend on its position in the uterine horn due to the inherent variation of the placental nutrients transporting capacity (Perry & Rowell, 1969). This is also the reason why there were still IUGR piglets in folic acid supplemental diet. However, despite the existence of IUGR piglets in all treatment groups, there was internal differences among those piglets characterized by gene expressions, and hence possible phenotype changes.

In the present study, we characterized several key apoptosis-related proteins encoded genes between IUGR and NBW piglets, such as p53, Bcl-2, Bax, IGF, and found that these protein encoded genes were unfavorably affected by uteroplacental insufficiency, which was inconsistent with previously reported findings in rats (Pham et al., 2003). P53 has received great attention in the field of regulating cell apoptosis and tumorigenesis(Clarke et al., 1993; Miyashita & Reed, 1995), and up-regulated p53 expression was associated with increased cell apoptosis under chronic and acute stress (Cummings et al., 1996).

In the present study, p53 gene expression was increased by IUGR, indicating elevated cell apoptosis. This was evidenced by down-regulated Bcl-2 and IGF-1, and up-regulated Bax expressions. Bcl-2 and Bax were the downstream proteins of p53 and were responsible for the cell apoptosis. Bcl-2 and Bax could inversely activate caspase-3 activity to influence the chromatin condensation and DNA chain breaks, and consequently cell apoptosis (Porter & Nicke, 1999). The expression pattern of IGF-1 was affected by many factors including physiological stage, health status and nutritional adequacy, and fetal IGF-1 expression could be related to nutritional status (Thissen et al., 1994). Previous studies demonstrated that maternal nutrition inadequacy could decrease the fetal IGF-1 expression (Godfrey & Barker, 1995) which could indicate the intrauterine growth status. IGF-1 expression was decreased by IUGR, indicating this growth factor secretion was inhibited by the insufficient nutrient intake.

Upon realising the negative effects induced by IUGR, we proposed supplementation of folic acid as part of nutritional strategies to reverse these changes. We found that p53 and Bcl-2 gene expressions were improved by folic acid, but these effects were only observed for IUGR piglets, and not for NBW piglets, stressing the effectiveness of maternal folic acid supplementation as a solution against maternal nutritional constraints. However, folic acid

supplementation did not affect the IGF-1 and Bax expressions, indicating that IGF-1 and Bax was not sensitive to folic acid administration, or they are controlled by other pathways. Further research is needed to explain this observation.

Previous studies found that alteration of p53 DNA CpG methylation was the potential mechanism mediating the IUGR on gene expressions and cell apoptosis (Pham et al., 2003). Therefore, we focused on DNMT-1, on which genomic methylation depends (Okano et al., 1999). The DNMT-1 was decreased in IUGR individuals compared to NBW individuals in the present study, which was consistent with previous study showing that the altered one-carbon metabolism represented one of the important characteristics in IUGR individuals compared to normal intrauterine growth (Pham et al., 2003; MacLennan et al., 2004). Folic acid, a key factor to modulate one-carbon metabolism and keep the genome methylation in normal status, could affect the gene expression of DNMT-1(Friso & Choi, 2002; Koutros et al., 2007). Striking evidence was also available to suggest folic acid as the potential nutritional therapy to the IUGR individuals since folic acid supplementation reversed the methylation status induced by maternal protein restriction (Lillycrop et al., 2005). In the present study, folic acid supplementation reversed the renal DNMT-1 expression change in IUGR piglets, and probably altered p53 gene expression subsequently.

Collectively, our study revealed that maternal folic acid supplementation could favorably reverse the gene expressions induced by IUGR. This result provides strong support for using folic acid as a potential nutritional "therapy" for reversing the negative effects induced by IUGR on renal gene expressions.

6

ACKNOWLEDGEMENTS: This work was supported by the Program for Changjiang Scholars and Innovative Research Team in University of China (IRT0555-5).

7 REFERENCES

Clarke A, Purdie C, Harrison D, Morris R, Bird C, Hooper M, and Wyllie A: 1993. Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature 362: 849-852.

Cummings M: 1996. Increased p53 mRNA expression in liver and kidney apoptosis. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease 1315(2): 100-104.

Friso S. and Choi S: 2002. Gene-nutrient interactions and DNA methylation. Journal of Nutrition 132(8): 2382S-2387S.

Gobe G, Zhang X, Willgoss D, Schoch E, Hogg N, and Endre Z: 2000. Relationship between expression of Bcl-2 genes and growth factors in ischemic acute renal failure in the rat. Journal of the American Society of Nephrology 11(3): 454-467.

Godfrey K. and Barker D: 1995. Maternal nutrition in relation to fetal and placental growth. European Journal of Obstetrics Gynecology and Reproductive Biology 61(1):15-22.

Hinchliffe S, Lynch M, Sargent P, Howard C, and Velzen D: 2005. The effect of

intrauterine growth retardation on the development of renal nephrons. BJOG: An International Journal of Obstetrics & Gynaecology 99(4):296-301.

Miyashita JC and Reed JC: 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 80(2): 293-299.

Koutros S, Zhang Y, Zhu Y, Mayne S, Zahm S, Holford T, Leaderer B, Boyle P, and Zheng T: 2007. Nutrients contributing to one-carbon metabolism and risk of non-Hodgkin lymphoma subtypes. American Journal of Epidemiology 167: 287-294.

Lillycrop K, Phillips E, Jackson A, Hanson M, and Burdge G: 2005. Dietary protein restriction of pregnant rats induces ???? and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. Journal of Nutrition 135(6): 1382-1386.

Lindemann M. and Kornegay E: 1989. Folic acid supplementation to diets of gestating-lactating swine over multiple parities. Journal of Animal Science 67(2): 459-464.

MacLennan N, James S, Melnyk S, Piroozi A, Jernigan S, Hsu J, Janke S, Pham T, and Lane R: 2004. Uteroplacental insufficiency alters DNA methylation, one-carbon metabolism, and histone acetylation in IUGR rats. Physiological Genomics 18(1): 43-50.

Okano M, Bell D, Haber D, and Li E: 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. CELL 99: 247-258.

Perry J. and Rowell J: 1969. Variations in foetal weight and vascular supply along the uterine horn of the pig. Reproduction 19(3): 527-534.

Pham T, MacLennan N, Chiu C, Laksana G, Hsu J, and Lane R: 2003 Uteroplacental insufficiency increases apoptosis and alters p53 gene methylation in the full-term IUGR rat kidney. American Journal of Physiology- Regulatory, Integrative and Comparative Physiology 285(5): 962-970.

Porter A. and Nicke R J: 1999 Emerging roles of caspase-3 in apoptosis. Cell Death and Differentiation 6(2): 99-104.

Thissen J, Ketelslegers J, and Underwood L: 1994. Nutritional regulation of the insulin-like growth factors. Endocrine Reviews 15(1): 80-101.

Wang J, Chen L, Li D, Yin Y, Wang X, Li P, Dangott L, Hu W, and Wu G: 2008. Intrauterine growth restriction affects the proteomes of the small intestine, liver, and skeletal muscle in newborn pigs. Journal of Nutrition 138(1): 60-66.

Wu G, Bazer F, Wallace J, and Spencer T: 2006. Intrauterine growth retardation: Implications for the animal sciences. Journal of Animal Science 84(9): 2316-2337.

Xu Y, Tang Y, and Li Y: 2007. Effect of folic acid on prenatal alcohol-induced modification of brain proteome in mice. British Journal of Nutrition 99(03): 455-461.