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Effects of folic acid supplementation on growth performance and hepatic folate metabolism-related gene expressions in weaned piglets

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Abstract The present study was conducted to evaluate the effects of different folic acid supplemental levels on growth performance, serum biochemical indicators, and hepatic folate metabolism-related gene expressions in weaned piglets. There were 160 piglets with initially average bodyweight of 7.33 kg randomly assigned to diets containing five levels of folic acid: basal diets (C), 0.5 mg·kg⁻¹ folic acid (FS 0.5), 2.5 mg·kg⁻¹ folic acid (FS 2.5), 5.0 mg·kg⁻¹ folic acid (FS 5.0), or 10.0 mg·kg⁻¹ folic acid (FS 10.0). Blood samples were collected from a subset ($n = 20$; 4 pigs per treatment) of the piglets on day 0, 14, and 28. Liver samples were collected from the blood-taken piglets on day 28 of the experiment. Pigs fed basal diet supplemented with 2.5 mg·kg⁻¹ folic acid grew faster ($P < 0.05$) and consumed more feed ($P < 0.01$) than groups of C, FS 5.0, and FS 10.0 during the last two weeks. Dietary treatment had no effect on F/G throughout the experiment. Pigs in the FS 2.5 group showed greater concentrations of Growth Hormone (GH) ($P < 0.05$) and Insulin-like Growth Factors (IGF-1) ($P < 0.01$) in serum than C and FS 10.0 on day 28. RT-PCR analysis revealed that FS 0.5, FS 2.5, and FS 5.0 had a greater abundance of the mRNA encoding 5,10-methylenetetrahydrofolate reductase than C and FS 10.0 ($P < 0.01$). The mRNA expressions of folate binding protein in FS 0.5 and FS 2.5 were upregulated compared with pigs fed with basal diet ($P < 0.05$). These results demonstrated that folate supplemental level of 2.5 mg·kg⁻¹ significantly enhanced the growth performance of piglets. Folic acid had an impact on

folate metabolism and the homocysteine concentrations. No folate supplementation or folate supplemental level of 10 mg·kg⁻¹ could not increase the growth performance to the greatest degree.

Keywords folic acid, growth performance, folate binding protein (FOLBP), 5,10-methylenetetrahydrofolate reductase (MTHFR), piglets

1 Introduction

Folic acid, which exists naturally in green vegetables as a synthetic form of vitamin B, plays a significant role in one-carbon metabolism and is essential for the synthesis of precursors of nucleic acids, and, hence, the synthesis of DNA (Lucock, 2000).

The liver is an important organ for folic acid metabolism. Methyl-tetrahydrofolate (CH₃-THF) is the active form of folic acid that connects the relationship between folic acid and methionine cycle. The maintenance of balance between folate and methionine cycle is vital for growth and development since folic acid is involved in the assistance of methylation action (Niculescu and Zeisel, 2002). Unfortunately, mammalian cells themselves are unable to couple the pteridine ring to other compounds for folate biosynthesis and are dependent on dietary folic acids (Birn, 2006).

The important role of folate cycle in cell metabolism could also be reflected by the close relationship with growth performance. Lévesque et al. (1993) reported that a diet supplemented with folic acid improved growth rate with no effect on feed intake of white veal calves. Matte et al. (1993) demonstrated that folic acid could influence growth performance of gilts at the end of the growing period. Few studies are documented on the effects of folic acid on weaned piglets' growth performance.

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Folic acid was traditionally considered safe due to its water-soluble character. However, some researches showed that high-level folate supplementation would decrease bodyweight and vertex-coccyx length in rat fetuses (Achón et al., 1999). It remains unclear whether the possible deleterious effects of high folic acid outweigh the known potential benefits in piglets.

In our study, the effects of different folic acid intake on growth performance of weanling piglets were investigated.

2 Materials and methods

2.1 Animals and experimental design

The experiment was designed by single factor random allotment. A total of 160 pigs (80 of Landrace and 80 of Yorkshire), initially 7.33 ± 0.67 kg and 25 days of age, were divided into five dietary treatment groups: (1) basal diet, (2) basal diet supplemented with $0.5 \text{ mg} \cdot \text{kg}^{-1}$ folic acid, (3) basal diet supplemented with $2.5 \text{ mg} \cdot \text{kg}^{-1}$ folic acid, (4) basal diet supplemented with $5.0 \text{ mg} \cdot \text{kg}^{-1}$ folic acid, and (5) basal diet supplemented with $10.0 \text{ mg} \cdot \text{kg}^{-1}$ folic acid.

2.2 Pigs and housing

All experimental procedures were approved by the Animal Care Advisory Committee of Sichuan Agricultural University. The pigs were reared in a nursery facility in groups of eight pigs per pen, with four replications per dietary treatment. All pigs had *ad libitum* access to feed and water through a self-feeder and a nipple water system.

2.3 Diets

All diets without antibiotics were formulated to meet or exceed the established nutrient requirements for weanling pigs. The pigs were fed for two dietary phases, with the pigs being switched to the second phase after the initial two weeks (Table 1).

2.4 Pig bodyweight and blood and liver sampling

Pigs were weighed on days 0, 14, and 28 and slaughtered at 8:00 in the morning at the end of the experiment. The pigs fasted for 8 h before blood sampling were weighed. Blood samples were collected from the precaval vein on days 0, 14, and 28. All blood samples were centrifuged at $3000 \times g$ for 15 min immediately after collection and stored at -20°C for analysis. After dissection, the liver samples were removed and cut into small pieces, washed with 0.9% physiologic saline, and immediately put into liquid nitrogen. Subsequently, the liver samples were stored at -80°C for RNA isolation.

Table 1 Composition and nutrient levels of basal diets (air-dry basis, %)

item	phase 1 (week 1–2)	phase 2 (week 3–4)
ingredient/%		
corn	46.50	52.70
soybean oil	4.25	4.10
extruded soybean	13.00	17.00
fish meal	4.50	4.00
plasma protein	6.00	3.00
fermented soybean meal	4.20	5.00
vitamin-mineral premix ¹	1.00	1.00
limestone	0.60	0.70
sodium chloride	0.46	0.32
dicalcium phosphate	0.70	0.70
whey powder	15.00	9.00
white sugar	3.00	2.00
L-lysine HCl	0.47	0.30
DL-methionine	0.21	0.07
L-threonine	0.27	0.13
zinc oxide	0.30	0.30
calculated analysis/%		
crude protein	20.06	18.99
metabolic energy/ $\text{Mcal} \cdot \text{kg}^{-1}$	3.398	3.399
Ca	0.74	0.71
total P	0.59	0.58
available P	0.42	0.39
lysine	1.60	1.35
methionine	0.53	0.40
methionine + cystine	0.80	0.67
threonine	1.04	0.86
folic acid (determined)	$0.48/\text{mg} \cdot \text{kg}^{-1}$	$0.41/\text{mg} \cdot \text{kg}^{-1}$

Note: 1: Vitamins and trace mineral premix are provided as follows (per kg of diet): vitamin A, 15000 IU; vitamin D₃, 1500 IU; vitamin E, 100 IU; vitamin K₃, 3 mg; vitamin B₁, 3 mg; vitamin B₂, 7 mg; vitamin B₆, 6 mg; vitamin B₁₂, 0.04 mg; Niacin, 40 mg; Pantothenic acid, 12 mg; Biotin 0.1 mg; Fe, 165 mg; Cu, 200 mg; Zn, 145 mg; Mn, 65 mg; Se, 0.18 mg; I, 0.38 mg. No folic acid was added in vitamin premix.

2.5 Analysis

Folate in serum and feed were determined using radioimmunoassay (SimulTRAC-SNB Folate Radioassay Kit from MP Biomedicals, Germany). Serum creatinine (CRE) concentration was analyzed using a Hitachi 7020 automated analyzer. Alkaline phosphatase (ALP) activity was measured at 405 nm by the formation of paranitrophenol from paranitrophenylphosphate as a substrate. Serum homocysteine concentration was determined by chemiluminescence method using a commercially available kit (Abbott Pharmaceutical Co., Ltd., USA) and performed by AXSYM automatic chemiluminescence instrument. Serum growth hormone (GH) and insulin (INS) concentration was

measured by chemiluminescence method using a commercial assay kit (Tosoh Corporation, Japan) and performed by AIA-1800 automatic fluorescent magnetic particle enzyme immunoassay analyzer. Serum insulin-like growth factor 1(IGF-1) concentrations were determined by extraction and RIA procedures.

2.5.1 RNA isolation and reverse transcription

RNAs were isolated from the liver of each piglets using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. The mRNA expression levels of folate binding protein (FOLBP) and 5,10-methylenetetrahydrofolate reductase (MTHFR) in liver were determined because the liver is the main site for folate metabolism and storage. Villanueva et al. (1998) demonstrated that FOLBP involved in folate homeostasis was regulated uniquely in pig liver plasma membranes and its transcriptional level was three times in liver than in kidney, and MTHFR was one of the most important enzymes in folate metabolism (Bailey and Gregory, 1999). Primers used for PCR and nested PCR for the detection of *MTHFR*, *FOLBP*, and *H2A* position on cDNA are shown in Table 2.

2.5.2 Statistical analysis

All data were analyzed using SPSS 13.0 by one-way ANOVA and multiple comparisons of Duncan analysis to determine statistical differences between groups. In all experiments, pen was considered as an experimental unit. An alpha level of $P < 0.05$ was used for the determination of statistical significance. The results were presented as the mean±SD.

3 Results

3.1 Growth performance

During the first phase of the test, there was no significant difference in growth performance between different folate supplemental levels, but pigs in FS 2.5 grew faster and consumed more feed than the other four groups numerically (Table 3). However, the effect of folic acid supplementation on ADG and ADFI began to be obvious

in the phase 2 of the experiment. During which, adding 2.5 mg·kg⁻¹ folic acid had a greater ADG ($P < 0.05$) and ADFI ($P < 0.01$) than C, FS 5.0 and FS 10.0. ADG over the entire four-week experimental period was greater ($P < 0.05$) in FS 2.5 than pigs fed with either C or FS 10.0. Consequently, body weight at the end of experiment was higher in FS 2.5 than the other groups ($P = 0.06$). Feed to Gain ratio was not affected in different groups.

3.2 Serum biochemical indicators

The effects of dietary folic acid supplemental levels on serum biochemical indicators were presented in Table 4. The concentration of GH in FS 2.5 was higher ($P < 0.05$) compared with C and FS 10.0 on day 28 of the test. In addition, FS 2.5 showed a greater ($P < 0.05$) IGF-1 concentration than C and FS 10.0 on day 14 of experiment. Furthermore, at the end of the test, the level of IGF-1 was extremely higher ($P < 0.01$) in FS 2.5 and was greater ($P < 0.05$) in FS 0.5 than C and FS 10.0. Serum insulin and creatinine and alkaline phosphatase concentrations were not affected by different folate supplemental levels ($P > 0.05$).

3.3 Serum folate and homocysteine

With the increased folic acid supplementation, serum folate concentration was enhanced apparently (Fig. 1). On day 14 of the test, FS 10.0 resulted in a significant higher folic acid level than C ($P < 0.01$), FS 0.5 ($P < 0.01$), and FS 2.5 ($P < 0.05$). At the end of the experiment, FS 2.5 showed greater folate concentration than C ($P < 0.05$) and FS 0.5 ($P < 0.05$). The homocysteine levels showed a negative correlation with the supplementation of folic acid (Fig. 2). At the end of experiment, the homocysteine concentrations in FS 2.5, FS 5.0 and FS10.0 were significantly lower than C and FS 0.5 ($P < 0.01$). However, there were no differences among FS 2.5, FS 5.0, and FS 10.0.

Figure 1 showed that serum folic acid concentrations were increased with the folic acid supplemental levels.

Figure 2 showed that serum homocysteine concentrations decreased with folic acid supplementation from 0.5 to 10 mg·kg⁻¹ at day 14 and from 0 to 2.5 mg·kg⁻¹ at day 28, but no further decline was observed for FS 2.5, FS 5.0, and FS 10.0 at the end of the experiment.

Table 2 Primers used for PCR and nested PCR for detection of *MTHFR*, *FOLBP*, and *H2A* position on cDNA

gene	primer sequences (5'–3')	annealing temperature/°C	product Tm	product size/bp	GenBank accession no.
<i>MTHFR</i>	F: AGACCATACTGCACATGACCTG	59.7	78.5	155	AF239166
	R: GTAGCTGAAGCCTCCTTCCTC				
<i>FOLBP</i>	F: TTGAGTCCTTCTTCCCCACAC	56	88	152	NM_213853.1
	R: GAACCTCGCTACCTCCTCGTT				
<i>H2A</i>	F: TGGATGTCCTTGGGCATG	60.1	91.5	226	BP459633
	R: AGATCCGGCGCTACCAGA				

Table 3 Effects of different levels of dietary folic acid on growth performance of weaned pigs

item	levels of folic acid				
	C	FS 0.5	FS 2.5	FS 5.0	FS 10.0
ADG/g					
Phase 1	242.63±20.79	243.30±18.07	263.62±20.06	245.98±29.89	242.97±15.32
Phase 2	459.26±41.05 ^b	487.72±25.57 ^{ab}	522.32±16.03 ^a	463.39±39.34 ^b	461.50±22.94 ^b
week 1 to 4	350.95±27.21 ^b	365.51±21.08 ^{ab}	392.97±21.32 ^a	354.69±28.88 ^{ab}	352.23±11.45 ^b
ADFI/g					
Phase 1	314.82±23.26	315.87±9.21	325.04±29.60	308.92±28.40	309.37±9.15
Phase 2	692.08±39.89 ^c	717.44±21.73 ^{ac}	761.98±22.46 ^a	694.02±47.99 ^c	693.08±19.19 ^c
week 1 to 4	503.45±29.46	516.66±14.73	543.51±21.77	501.47±37.83	501.22±29.72
feed:gain/ g/g					
Phase 1	1.29±0.12	1.30±0.07	1.24±0.04	1.26±0.04	1.28±0.05
Phase 2	1.51±0.05	1.47±0.04	1.46±0.03	1.50±0.07	1.50±0.04
week 1 to 4	1.44±0.04	1.42±0.05	1.38±0.03	1.42±0.05	1.43±0.03
BW/kg					
initial	7.320±0.69	7.325±0.67	7.325±0.73	7.331±0.73	7.361±0.64
week 4	17.147±1.39	17.559±1.15	18.328±1.78	17.263±1.69	17.223±1.38

Note: Data are presented in mean±SD. Mean values with different superscripts in the same rows are significantly different at $P < 0.05$, the interval of letter sequence gapped two or more differed at $P < 0.01$. Phase1 diets were fed for weeks 1 and 2, and phase 2 diets were fed for weeks 3 and 4.

3.4 Expression of hepatic *FOLBP* and *MTHFR*

3.4.1 *FOLBP*

As shown in Fig. 3, dietary folic acid supplemental levels significantly affected ($P < 0.05$) the expression of *FOLBP*. The transcript expression level of *FOLBP* was higher ($P < 0.05$) in FS 0.5 and FS 2.5 than C.

3.4.2 *MTHFR*

There were significant differences ($P < 0.01$) in *MTHFR* expression among treatment groups (Fig. 4). The expression of *MTHFR* was higher ($P < 0.01$) in FS 0.5, FS 2.5 and FS 5.0 than C and FS 10.0.

4 Discussion

Folic acid is an essential nutrient for animal growth and development as an important member of the vitamin B family. Limited reports of folic acid influencing growth performance of weaned-piglets showed no consistency. Newcomb et al. (1986) and Easter et al. (1983) found that there was no significant effect on growth performance of piglets, which was in consistent with Lindemann and Kornegay's (1986) study that adding 0.5 mg·kg⁻¹ folic acid could improve ADG of piglets. However, as presented in Table 3, basal diet supplemented with 2.5 mg·kg⁻¹ folic acid could increase ADG and ADFI in the final two weeks compared with other treatments. Either inadequate or excessive intake of folic acid was not good for achieving

the maximal growth potential of weaned piglets.

The growth of an organism depends on an increase in the number and size of existing cells. The rapid growth of the weaned piglets requires high rate of cell turnover that has to meet with synchronization of DNA metabolism. Folic acid participates in one-carbon metabolism and plays a vital role in the synthesis of DNA precursors. Therefore, sufficient folic acid must be provided into animal diet to guarantee fundamental requirement for body metabolism. However, the overintake of folic acid might bring about unfavorable effects on normal folate cycle. Folic acid is transported into the cell through a receptor-mediated process by *FOLBP*, which is a high-affinity folate binding protein. In the present study, we observed that the mRNA expression of *FOLBP* were downregulated in response to 5 mg·kg⁻¹ and 10 mg·kg⁻¹ of folic acid supplemental level. Decreased expression of *FOLBP* might result in the reduction of folic acid entering the cell to take part in folate metabolism. *MTHFR* is one of the most important enzymes catalyzing 5,10-methylenetetrahydrofolate to synthesize 5-methyltetrahydrofolate, which is the major carbon donor in remethylation of homocysteine to methionine that is involved in DNA synthesis and cell metabolism. In this study, the mRNA expression of *MTHFR* was upregulated in response to 0.5 mg·kg⁻¹, 2.5 mg·kg⁻¹, and 5 mg·kg⁻¹ of folic acid supplemental level. There was a significant quadratic relationship ($P < 0.05$) between *MTHFR* mRNA expression and folate supplemental levels. Earlier studies found that high-dose folic acid reduced the activity of *MTHFR* because of dihydrofolate serving as a folate antagonist and a potent inhibitor of *MTHFR* (Matthews and Baugh, 1980). Similar

Table 4 Effects of levels of folate on serum biochemical indicators of weanling pigs

item	levels of folic acid				
	C	FS 0.5	FS 2.5	FS 5.0	FS 10.0
GH/g·L ⁻¹					
days of postweaning					
0	1.22±0.04	1.26±0.03	1.23±0.06	1.25±0.06	1.26±0.11
14	1.39±0.08	1.48±0.12	1.48±0.10	1.46±0.15	1.43±0.06
28	1.48±0.12 ^b	1.57±0.08 ^{ab}	1.59±0.17 ^a	1.51±0.07 ^{ab}	1.49±0.05 ^b
IGF-1/ng·mL ⁻¹					
days of postweaning					
0	87.65±6.18	88.65±3.46	89.29±4.03	88.66±4.51	89.97±2.56
14	105.46±14.65 ^b	113.86±16.42 ^{ab}	117.94±11.19 ^a	113.34±15.37 ^{ab}	106.71±10.32 ^b
28	130.95±16.90 ^c	148.27±17.90 ^{ab}	151.18±15.38 ^a	143.00±11.98 ^{abc}	132.36±16.72 ^c
INS/IU·L ⁻¹					
days of postweaning					
0	21.40±1.69	21.94±1.71	22.02±2.16	23.15±1.80	21.65±2.00
14	29.94±1.91	30.76±1.61	31.25±0.43	31.42±0.97	30.35±0.83
28	32.06±1.90	34.61±1.61	33.95±1.27	33.76±1.55	33.99±2.77
creatinine/mol·L ⁻¹					
days of postweaning					
0	94.73±10.14	96.68±9.09	97.27±11.82	96.51±9.89	98.52±9.15
14	87.96±9.59	86.90±9.60	85.36±10.20	84.74±8.47	85.94±9.93
28	78.82±9.07	83.79±8.44	80.63±9.39	79.50±8.54	80.18±10.26
alkaline phosphatase/U·L ⁻¹					
days of postweaning					
0	334.60±40.57	347.23±24.68	333.58±35.55	342.07±39.42	348.28±29.58
14	287.13±22.68	293.05±34.58	294.45±21.88	296.10±31.99	305.18±32.64
28	293.15±22.33	303.18±32.09	307.58±35.63	302.48±37.56	302.53±26.37

Note: Data are presented in mean±SD. Mean values with different superscripts in the same row are significantly different at $P < 0.05$, interval of letter sequence gapped two or more differed at $P < 0.01$.

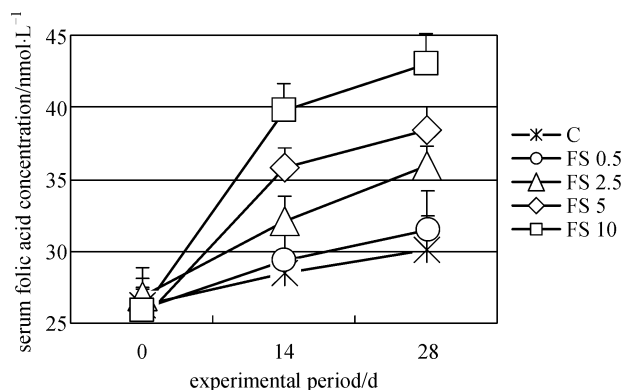


Fig. 1 Serum folic acid concentrations in piglets in response to different folate supplemental levels

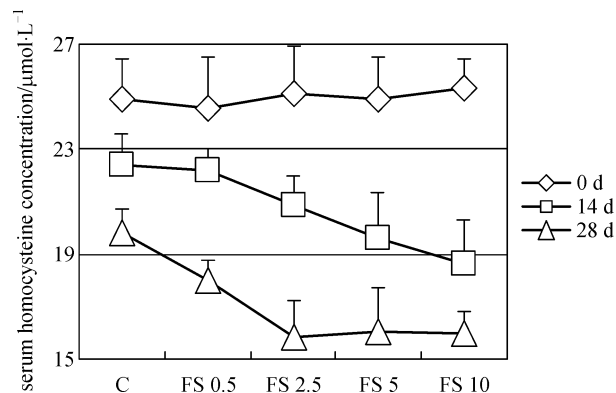


Fig. 2 Serum homocysteine concentrations in piglets in response to different folate supplemental levels

results were observed in human studies. Ashokkumar et al. (2007) reported that the downregulation in folate uptake in the folate-oversupplemented cells was associated with a decrease in the mRNA and protein levels of folate receptor.

The present study demonstrated that folic acid supplementation should be at appropriate dosage, since folate inadequacy or overconsumption would disturb folic acid cycle and, hence, cell metabolism.

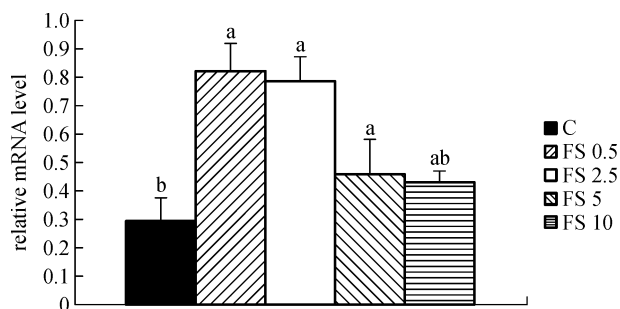


Fig. 3 Effect of folate supplemental levels on the relative expression of *FOLBP* gene in livers of piglets

Note: The transcript expression level of *FOLBP* was higher ($P < 0.05$) in FS 0.5 and FS 2.5 than C. Data are presented in mean $\pm$ SD. Different small letters denote statistical differences between groups ($P < 0.05$).

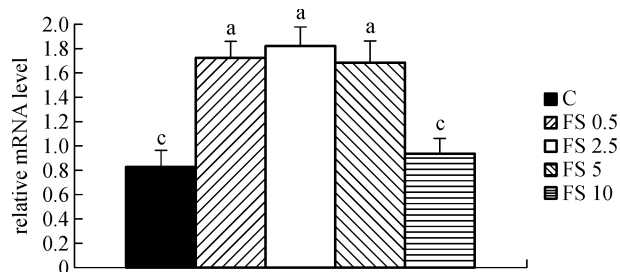


Fig. 4 Effect of folate supplemental levels on the relative expression of *MTHFR* gene in livers of piglets

Note: Higher ($P < 0.01$) expression was observed in FS 0.5, FS 2.5, and FS 5.0 than C and FS 10.0. Data are presented in mean $\pm$ SD. Different small letters denote statistical differences between groups ($P < 0.05$).

In the present study, homocysteine concentrations were reduced with increased folic acid supplemental levels, but at the end of the experiment, serum homocysteine concentrations were not significantly reduced. Homocysteine Lowering Trialists' Collaboration (2005) also found that daily doses of folic acid greater than 0.8 mg could achieve the maximal reduction in plasma homocysteine concentration of humans. This is partly due to the attenuated folate cycle represented by a downregulated expression of *FOLBP* and *MTHFR*. Other mechanisms cannot be ruled out because the low expression of *FOLBP* and *MTHFR* should have reduced cellular folic acid and hence lead to higher homocysteine concentration. Convincing experiments are needed to elucidate this issue.

The rapid growth rate of piglets after weaning mainly depends on protein deposit rather than adipose tissue. To date, there were few studies that addressed the direct relationship between folic acid and protein metabolism. However, indirect evidences suggested that folic acid might be associated with protein synthesis. First, folic acid is involved in the interconversion of several amino acids, such as serine and glycine, histidine and glutamic acid,

methionine, and homocysteine. Folic acid, mainly in the active form of tetrahydrofolate, plays a role as one-carbon carrier to contribute to the synthesis of pyrimidine and purine, and, thereby, protein synthesis through central dogma. Second, Stern et al. (2004) elucidated that homocysteine had detrimental effects on protein metabolism, and it would accelerate protein degradation rates, and homocysteine might interact with nucleic acid thus disturbed protein synthesis. Herein, we observed that GH and IGF-1, hormones that maintain protein synthesis, differed in response to folate supplemental levels. In particular, serum IGF-1 concentration was significantly negatively correlated ($P < 0.05$) with excessive dietary folate supplemental levels. The reason of the changed hormone secretion appeared to be linked to homocysteine homeostasis. Sesmilo et al. (2001) revealed that the acceleration of homocysteine metabolism resulted in increased levels of methionine and cysteine that was associated with protein synthesis, thus enhancing GH concentration. Besides, variations in homocysteine were negatively related to changes in IGF-1. Another hypothesis is that folic acid could act directly as the ancillary agonist of N-methyl-D-aspartate receptor stimulating growth hormone releasing hormone (GHRH) gene expression in hypothalamus, promoting GHRH synthesis and secretion, then accelerating GH synthesis and secretion in adenohypophysis, since a positive correlation was found to exist between GH and NMDA receptor (Le Greves et al., 2002). N-methyl-D-aspartate receptor agonists protecting homocysteine-induced developmental abnormalities also support this opinion (Rosenquist et al., 1999).

5 Conclusion

The folate supplemental level of 2.5 mg $\cdot$ kg⁻¹ significantly enhanced the growth performance of piglets. Folic acid had an impact on folate metabolism and the homocysteine concentrations. None folate supplementation or folate supplemental level of 10.0 mg $\cdot$ kg⁻¹ could not increase the growth performance to the greatest degree.

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